University of Alberta

Systematics of the Spirorbinae (Annelida, Polychaeta, Serpulidae): The evolution of miniaturization, brooding modes, and coiling asymmetry

by

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ABSTRACT

The Spirorbinae are ubiquitous and diminutive polychaetes that have distinctive spirally-coiled calcareous tubes (<5 mm across spiral). They have unique morphological traits whose evolution may shed light on topics that have intrigued biologists for decades. For instance, their asymmetric bodies and diverse brooding modes may hold clues about the role of developmental plasticity in evolution and the origin of morphological novelties. Addressing such questions requires a phylogenetic framework. Relationships among spirorbin tribes (delineated based on brooding mode) were elucidated using morphological and molecular (18S and 28S rDNA) data. Although basal relationships among tribes had low support, unexpected patterns emerged: (1) brooding in a modified operculum (plug-like tentacle) - long thought to be derived within the Spirorbinae - is ancestral and likely evolved twice, (2) brooding inside the tube arose independently at least three times from opercular-brooding ancestors, (3) body size *increase* in spirorbin lineages is more common than size *decrease* and (4) dextral coiling appears to be prone to reversals, whereas sinistral spirorbin lineages tend to remain sinistral. Thus these assumptions about evolution in the Spirorbinae need to be re-evaulated. Two issues are further explored: costs and benefits of brooding modes, and the nature of asymmetry in dimorphic species. Comparison of numerous life history traits among ten spirorbin species reveal intriguing differences among tube- and opercular-brooders: (1) tube brooders have smaller minimum brooding body sizes and (2) renewing an opercular brood chamber may accrue a cost in brood turnover time. The second assumption addressed was the nature of dimorphism in *Paradexiospira vitrea* (which coils both left and right in Barkley Sound). Although otherwise identical, all evidence - phylogenetic, genetic, and ecological – points to sinistral and dextral *P. vitrea* being ecologically and genetically isolated lineages. This finding calls into question reports of *situs inversus* in other species, and highlights the need for an integrative approach to taxonomy.

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I could start no other way than with thanking my advisor, Dr. Rich Palmer. His enthusiasm for all things 'cool', and his willingness to take risks and pursue mysteries has inspired me. The thrill of discovery is so easily forgotten in the haze of deadlines and experiments, but his zeal never seems to wane. For his calming presence, I am grateful, and to his wisdom I am much indebted. I thank him for forgiving my time wasting, as I have learned to forgive myself.

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Graduate school has been an important journey. I learned not only to become a scientist, but also to become more confident, outgoing, and more trusting of my abilities. I learned to teach, to speak, and to question. I had the opportunity to see some far corners of the world. The following pages are only small portions of my learning experience.

DEDICATION

I dedicate this thesis to my parents, Valerie and Robert Macdonald, who have passed on to me a fascination for the sea and its inhabitants. My mother instilled in me at a young age a fascination with marine critters in general, and, later polychaetes in particular. She provided me with opportunity to learn about their diversity and thus develop an interest in systematics. My father had the sense to challenge my decision to follow in her footsteps, and in the end was my strongest supporter.

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CHAPTER 1.

THE SPIRORBINAE – MORPHOLOGY, ECOLOGY AND EVOLUTION

1.1 GENERAL INTRODUCTION

The Spirorbinae (Annelida, Polychaeta, Serpulidae) Chamberlain, 1919 are ubiquitous members of rocky intertidal communities worldwide. Their tightly coiled calcareous tubes and small size (<5 mm across tube whorls) (Fig. 1-1A) make them very distinctive, and readily apparent even to the untrained eye. These diminutive coiled tubes can be found on many substrates, including rocks, shells, various algae or angiosperms (*e.g.*, Fig. 1-1B), bryozoans, hydrozoans, crustaceans and molluscs. Many species occur in high densities (10-20/cm^2 (Daly 1978a, pers. obs.)), which may result from the gregarious settlement of brooded larvae (Knight-Jones 1951; Wisely 1960; Gee & Knight-Jones 1962, Nott 1973; Al-Ogily 1985; Fig. 1-1C). Thus the Spirorbinae are characterized not only by their unique form, but also their geographically widespread and aggregative distributions, high local abundances, and species diversity.

Spirorbin polychaetes are an intriguing group of tubeworms for many reasons. Their small size, conspicuously asymmetric body plan, and diverse assortment of wholly novel modes of brooding have piqued the curiousity of many biologists. These fascinating morphological traits may provide clues to more general evolutionary processes and the origin of novel forms. However, we know little about their phylogenetic relations, which greatly limits our understanding of how some of these interesting features of their biology evolved. Another unknown is how body size affects brooding in these tiny worms, and what relation brooding modes have to their overall fecundity; or how direction of coiling is inherited in dimorphic species. This thesis addresses some of these outstanding questions.

Spirorbin polychaetes belong in the family Serpulidae Latreille, 1825 ('fanworms'), which are well-known because of their colourful radiolar crowns (Fig. 1-1D) and meandering, uncoiled calcareous tubes (Fig. 1-1E). Spirorbins form a monophyletic clade within this family, which is strongly supported by morphological and molecular evidence (Pillai 1970, Macdonald 2003, Macdonald & Rouse *in review*, Kupriyanova *et al.* 2006, Lehrke *et al.* 2006). The Spirorbinae are unique among the Serpulidae because of their asymmetric body plans (Fig. 1-1F), miniaturized bodies and novel modes of incubating their young.

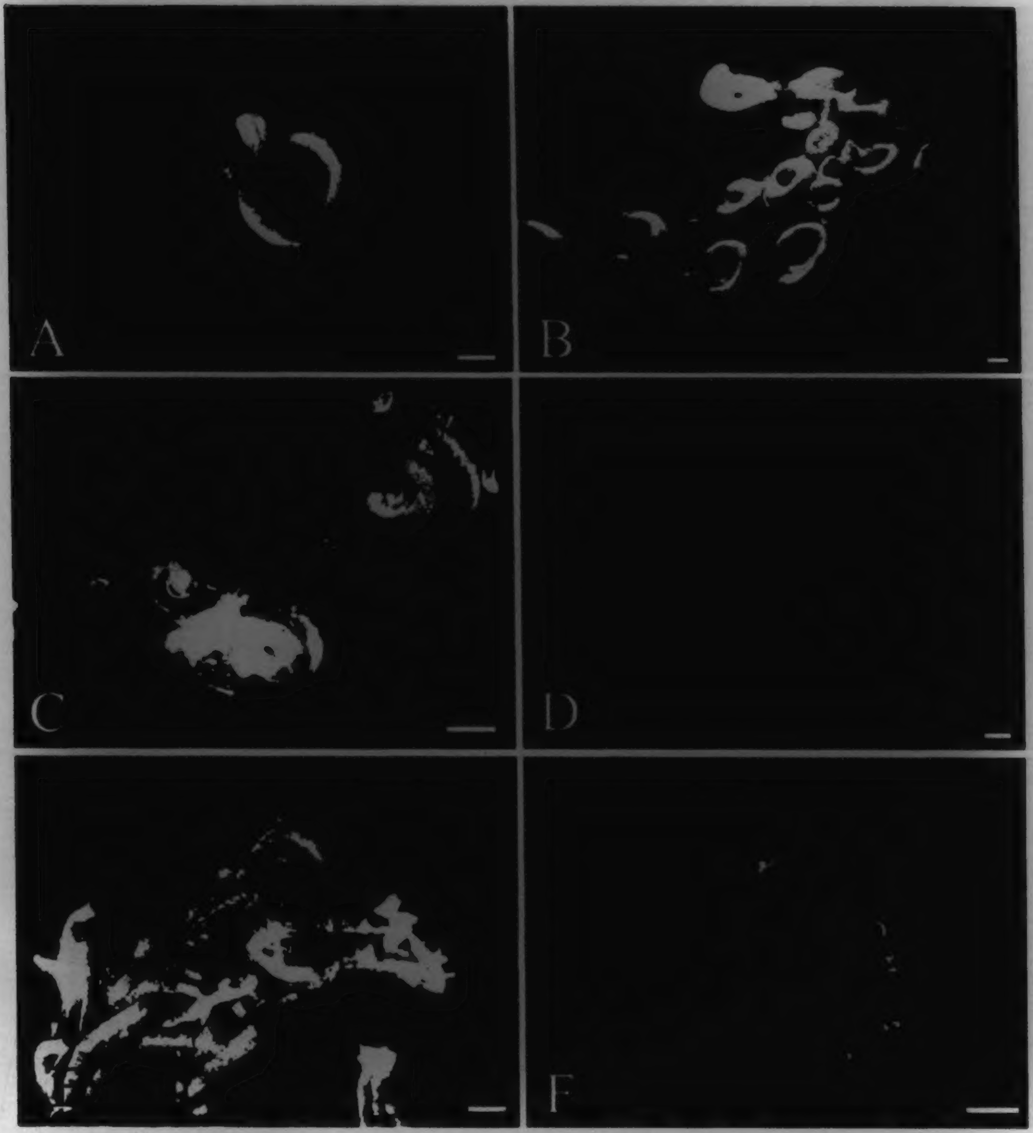

Fig. 1-1 Examples of serpuliform polychaetes. **A**. *Circeis armoricana* (Spirorbinae), **B**. *Eulaeospira orientalis* (Spirorbinae), **C**. *Paradexiospira vitrea* (Spirorbinae), **D**. *Crucigera zygophora* (Serpulinae), **E**. *Salmacina dysteri* (Filograninae), and **F**. *Paradexiospira vitrea* (Spirorbinae) removed from its tube. All species are from Barkley Sound, British Columbia with the exception of *E. orientalis* from Encounter Bay, South Australia. Scale bars 1 mm.

1.2 MORPHOLOGY AND PHYSIOLOGY OF THE SPIRORBINAE

1.2.1 Tube morphology

The most distinguishing characteristic of spirorbin polychaetes is their coiled calcareous tubes (a synapomorphy of the subfamily). Although these can vary in texture and sculpturing, they are easy to recognize because of their small, tightly coiled form. Tubes can be chalky and thick-walled, porcellanous and brittle, or glass-like (vitreous) (*e.g.,* Knight-Jones *et al.* 1979). They may have ornamentation such as longitudinal ridges, transverse ridges ('growth rings'), surface pits, or peripheral flanges (which presumably improve attachment; Rhzavsky 1994). Tubes of the Spirorbinae never exceed 5 mm in spiral diameter (across all whorls), but are usually quite uniform in size, approximately 2-3 mm in spiral diameter (*e.g.*, Knight-Jones & Fordy 1979).

The tube is formed from a mixture of calcium carbonate interspersed with a mucopolysaccharide matrix (Nott 1973). Only newly settled juveniles can secrete a tube *de novo*; subsequent tube growth requires the anterior extension of an existing tube. Upon metamorphosis, juveniles attach themselves to the substratum by secretion of a primary tube of organic material (Nott & Parkes 1975, Capizo-Ituarte & Hadfield 1998). They add to this tube by precipitating calcium carbonate in their peristomial tube glands, suspending these crystals in an organic matrix, and then molding this slurry onto the tube mouth (Hedley 1956, Neff 1971). All evidence indicates this process is similar in all serpuliform polychaetes, although how this process is modified in spirorbins to create a spiral tube remains unknown.

1.2.2 Body plan and chaetation

Like all members of order Sabellida, the Spirorbinae have distinct thoracic and abdominal regions (Fig. 1-2A). Unlike other members of the Sabellida, however, their thoracic region is often asymmetric with respect to the number of segments (more on the concave side) and number of chaetae (more on the concave side). Spirorbin tubeworms also have an additional body region, termed the achaetigerous region, which can be of significant length (up to 10 times the width of the last thoracic segment) (Fig 1-2A). The number of thoracic segments is a diagnostic taxonomic character among genera (Knight-Jones & Fordy 1979).

Polychaete taxonomy has historically relied on the form of chaetae, or chitinous bristles (Rouse & Pleijel 2001). All Serpulidae, including the Spirorbinae, have specialized chaetae in their first segment termed collar chaetae (Fig. 1-2Bi). These usually differ in morphology from other thoracic chaetae, which have long shafts and limbate blades (Fig. 1-2Biii). Collar chaetae are always notochaetae (dorsal chaetal bundle), and this first segment always lacks neurochaetae (ventral chaetal bundle). All other thoracic notochaetae are associated with neurochaetae; in the thorax, neurochaetae are uncini (reduced comb-like chaetae modified for gripping tube walls; Fig. 1-2Bvi). In the abdomen, this arrangement is reversed: the notochaetae are uncini, and the neurochaetae are 'normal' limbate chaetae (Fig. 1-2Bv). This arrangement is called 'chaetal inversion' and is a synapomorphy of the clade Sabellida (Rouse & Pleijel 2001).

The Serpulidae (Spirorbinae, Serpulinae and Filograninae) also possess a unique plug-like tentacle in their radiolar crown, whose morphology is an important taxonomic character of the Spirorbinae (Knight-Jones & Thorp 1984; Fig. 1-2C).

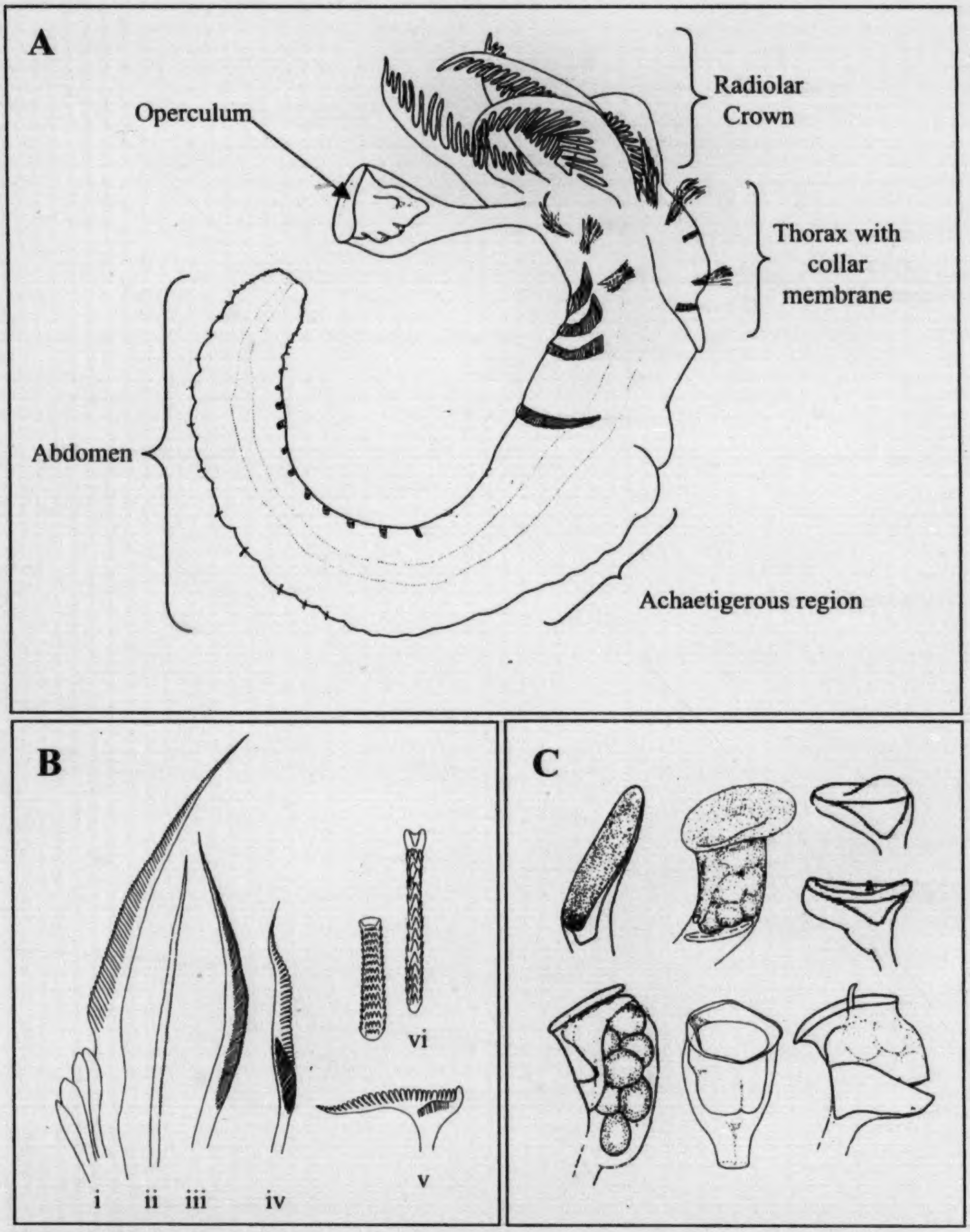
A
Operculum
Radiolar Crown
Thorax with collar membrane
Abdomen
Achaetigerous region
B
vi
v
i
ii
iii
iv
C

Fig. 1-2 Body plan and important taxonomic characters of the Spirorbinae. **A** Body plan of a generalized spirorbin. **B** Types of spirorbin chaetae; i) collar, ii) capillary, iii) limbate, iv) sickle, v) abdominal, vi) abdominal and thoracic uncini (left and right respectively). **C** Examples of opercular morphology in the Spirorbinae adapted from Knight-Jones & Thorp 1984.

1.2.3 Radiolar crown

All members of order Sabellida have a radiolar crown. It originates from the prostomium, or the first presegmental region of polychaetes (Rouse & Pleijel 2001). In spirorbin polychaetes, each half of the crown has a complement of approximately four radioles, but this is often greater in larger-bodied species (6-10 per side). On the concave side of the body, on the second dorsalmost radiole, spirorbins have an operculum, or modified plug-like tentacle (a characteristic they share with some serpulids). This tentacle presumably protects the worm from desiccation or predation (Thorp 1975, Thorp & Segrove 1975), and in some groups is also used as a brood chamber (*e.g.*, Bailey 1969, Knight-Jones *et al.* 1972). The operculum is often calcified and armoured. These ornamentations have been used as taxonomic characters; however, evidence of extensive phenotypic plasticity calls their taxonomic value into question (*e.g.*, Beckwitt 1981, Rhzavsky & Knight-Jones unpubl.).

1.2.4 Feeding, respiration, circulation and excretion

The radiolar crown is responsible for both feeding and respiration. Each radiole is pinnulated and ciliated and highly vascularized. Respiration is likely augmented by water currents that pass through the tube (Knight-Jones & Fordy 1979). Collar membranes have dense capillary beds to accommodate gas exchange across their surfaces (Hanson 1950, Knight-Jones 1981).

Spirorbin tubeworms filter feed on small particles, and can often be sustained strictly on bacteria in a laboratory setting (Potswald 1968), although in nature their guts are filled with small single-celled algae as well (pers. obs.). The esophagus and alimentary canals are simple ciliated tubes (Hanson 1949). The faecal groove is a ciliated tract that begins on the ventral abdomen, and curves gradually around the convex side of the asetigerous region to the dorsal side, where the faeces are expelled (Knight-Jones 1981).

The Spirorbinae, like all polychaetes, have closed circulatory systems. Hanson (1950) reviewed circulation in Serpulidae; although little is known about the circulation process in spirorbins specifically, we can assume it is similar to that of serpulids in general. In serpulids, a single ventral vessel carries the blood posteriorly. Blood moves anteriorly from the abdomen through a sinus that surrounds the alimentary canal, and then passes through a series of vessels in the thorax. Blood motion is presumably through peristalsis (of all mostly the abdominal segments), or perhaps the constriction of myoepithelial cells of the circulatory system. There is a series of contractile peripheral vessels, one of which serves the radiolar crown. The only known blood pigment in Serpulidae is chlorocruorin (Weber 1978).

Excretion is via a single pair of mixonephridia (Goodrich 1945) at the anterior end of the thorax. Some serpulids may also have a pair of ciliated ducts to the exterior in abdominal chaetigers, which may be strictly coelomoducts (through which gametes are expelled), but may have a nephridial component (Bartolomeus 1999).

1.2.5 Sense organs

The structure of serpulid nervous systems and sense organs were reviewed by Orrhage (1980). Again, no specific investigations into the nervous system morphology of the Spirorbinae have been done, but we can assume similarity to the Serpulidae. They have a complex brain compared to other tubiculous groups. They possess nuchal organs, which are a single pair of chemosensory ciliated pits that are considered to be a synapomorphy of polychaetes (Rouse & Fauchald 1997). In serpuliform polychaetes, these are internalized (Rouse & Pleijel 2001). Adult serpulids may have eyespots, which are readily apparent in larval stages of all serpulids (Kupriyanova *et al.* 2001), although their ultrastructure remains unknown.

1.2.6 Reproduction

All species of Spirorbinae are simultaneous hermaphrodites. Eggs are produced in the anterior 2-3 segments of the abdomen, and sperm in the remaining abdominal segments (Bergan 1953; Potswald 1967; King *et al.* 1969). Spermatids are released in clusters, tetrads, or eights, and are stored from a very young age in a single spermatheca at the base of the radiolar crown (Daly & Golding 1977, Picard 1980). Fertilization is internal; presumably taking place inside the tube but external to the body (Potswald 1967, 1968, Gee & Williams 1965).

All spirorbin tubeworms brood their young. Embryos are anchored in various ways (Bailey 1969, Knight-Jones *et al.* 1972; Fig. 1-3) and the subfamily is divided into six tribes based on brooding mode. I elect to call these groups tribes (instead of subfamilies under the family Spirorbidae) for reasons discussed below. Four of the six tribes brood inside their tubes: embryos of the Paralaeospirini (formerly Paralaeospirinae) are brooded loose inside the tube, embryos of the Circeini (Circeinae) adhere to the inside of the tube wall in a gelatinous matrix, embryos of the Spirorbini (Spirorbinae) are held in chains that attach to the posterior tube by a filament of tissue, and brood masses of the

Romanchellini (Romanchellinae) are attached to a thoracic brood stalk, which may be homologous with a recessed tentacle (Knight-Jones & Thorp 1984).

The remaining two tribes brood in opercular brood chambers - Pileolariini (formerly Pileolariinae) and Januini (Januinae). Their brood chambers are morphologically distinct, both developmentally and structurally (Knight-Jones & Thorp 1984). The brood chamber of the Januini is a cuticular cylinder enclosed by the primary opercular plate above, and a new opercular plate below the embryos. It is formed by the swelling and subsequent degeneration of the epithelial lining of the opercular ampulla. It is used for only one brood, as larvae are released by dehiscence of the brood chamber at its base, where the cuticular chamber joins with the living tissue surrounding the new opercular plate. The Pileolariini, on the other hand, have a brood chamber that can be used for more than one brood. It is formed by the invagination of the opercular ampulla and release of the pimary opercular plate (Thorp 1975 Thorp & Segrove 1975, Knight-Jones & Thorp 1984). The brood chamber walls are thus composed of a double layer of epithelium. They have a pore for the exit of larvae, and possibly the entrance of fertilized eggs. How these fertilized eggs enter the opercular brood chambers of both Pileolariini and Januini has stimulated much debate (Vuillemin 1965, Potswald 1968, 1977, Bailey 1969, Thorp 1975, Thorp & Segrove 1975, Knight-Jones & Thorp 1984).

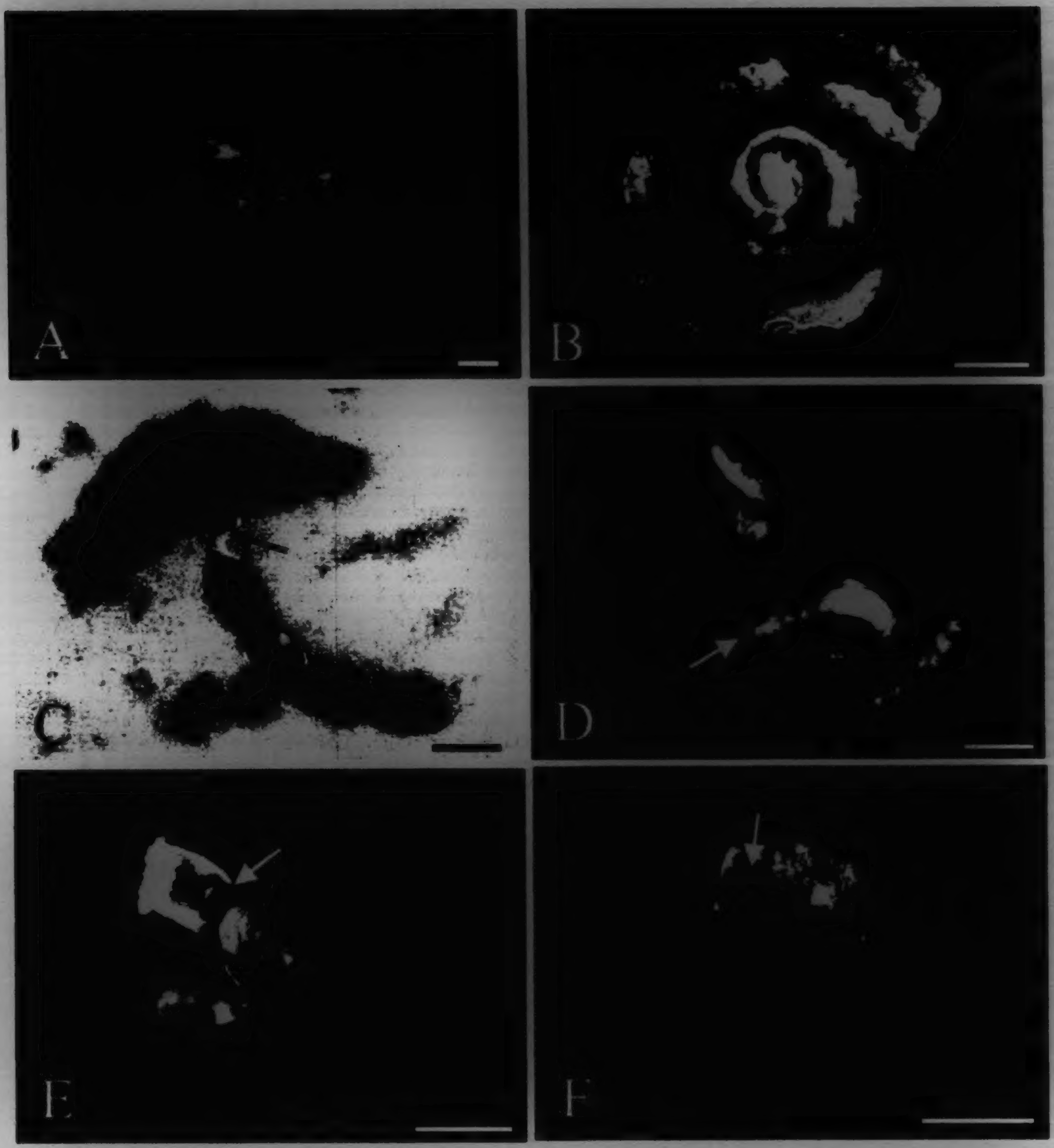

Fig. 1-3 Brooding modes of the Spirorbinae. **A.** *Eulaeospira convexis* (S. Australia) with its loose embryo string (Paralaeospirini); **B.** *Paradexiospira vitrea* (Barkley Sound, BC) with embryos adhering to the inner tube wall (Circeini), **C.** Egg string of *Spirorbis bifurcatus* (Spirorbini; Barkley Sound, BC) removed from tube; arrow indicates posterior attachment filament. **D.** *Protolaeospira eximia* (Romanchellini; Barkley Sound) removed from its tube; arrow indicates thoracic brood-stalk. **E.** *Neodexiospira* sp. (Januini) from S. Australia after embryo release; arrow indicates site of dehiscence of brood chamber. **F.** *Pileolaria pseudomilitaris* (Pileolariini) from S. Australia with opercular brood chamber containing embryos (arrow). Scale bars 1 mm except for C, 100μm.

Spirorbin tubeworms have lifespans that rarely exceed one year (Rhzavsky & Britayev 1984, Bergan 1953, Daly 1978a) and therefore have only one major reproductive season. Individuals appear to have approximately 3-5 sequential broods per season before senescing (Daly 1978a&b, Potswald 1967, 1968), although the number of broods and their timing remain relatively unexplored.

Larvae are brooded until they reach competence at approximately one month of age depending on temperature (Thorp 1991, Knight-Jones 1951, Daly 1978a). At this stage, they have three chaetigers and a terminal segment, bands of locomotory cilia, apical cilia, eyespots, branchial and opercular buds, and a large collar (Kupriyanova *et al.* 2001). Once liberated from the brood chamber, settlement usually occurs within a few hours after a brief pelagic stage (Knight-Jones 1951, Wisely 1960, Nott 1973, de Silva & Knight-Jones 1962). The duration of this stage depends on the presence of suitable substrate (Knight-Jones 1951, 1953), and may be eliminated altogether (Al-Ogily 1985). Settlement is often associative (*e.g.*, on macrophytes, bryozoans, hyrdrozoans, molluscs, and crustaceans) and gregarious (Knight-Jones 1951, de Silva 1962, Stebbing 1972, Ryland 1972).

Reproductive data exist only for Northern hemisphere Spirorbinae; these tend to have a reproductive peak in summer and autumn, with the breeding season extending from April-November depending on the species (Daly 1978b, Bergan 1953). Others are reproductive year-round, although the proportions of spawning individuals drops markedly in winter (Potswald 1967, Bergan 1953). While data for Southern hemisphere spirorbins are lacking, it appears tropical species are reproductive year-round (*e.g.*, observations in taxonomic papers such as Vine 1977), and the peak of breeding in austral/Antarctic species is in the austral summer (December-March) (Kupriyanova *et al.* 2001).

Since the Spirorbinae are short-lived, their brooding mode could have a significant impact on fecundity with respect to differences in brood duration and brood turnover. However, most studies of spirorbin reproduction have not been comparative but focused on single species, and often a single population of these. Thus it is difficult to say what differences exist in the life history traits among species with different brooding modes. The differences among ten spirorbin species encompassing the diversity of brooding modes with respect to brooding period, frequency and fecundity are investigated in Chapter 4.

1.3 NATURAL HISTORY OF THE SPIRORBINAE

1.3.1 Distribution and dispersal

Spirorbin polychaetes have a worldwide distribution, and are known from all oceans and continents. They are common in intertidal zones, but also occur subtidally, often to abyssal depths (*e.g.*, Zibrowius 1972). They are most thoroughly described from the Atlantic Ocean, especially in the United Kingdom and Europe owing to the work of Drs. P. and E.W. Knight-Jones (University of Wales, Swansea). In other parts of the world, such as the East Coast of North America, the entire coastline of Australia, Antarctica and the Arctic, and especially South America, have fewer, if any, published taxonomic surveys of spirorbin polychaetes have been done. In these places, species that fit no description are common (pers. obs.). Thus the approximately 140 described species of the Spirorbinae (Kupriyanova *et al.* 2001) is certainly an underestimate.

Contributing to this uncertain taxonomy is their lecithotrophic life history, and the resulting small-scale dispersal and increased genetic variability among populations (Strathmann 1985, Burton 1997). Cosmopolitan, widely distributed species, such as *Paradexiospira vitrea*, *Janua pagenstecheri* or *Circeis spirillum*, have been reported from all continents and oceans. However, their worldwide distribution seems at odds with their non-dispersive life history, and cryptic species may be more common than we currently believe (Packard & Taylor 1997). But brooding is not necessarily associated with limited geographic distribution in all taxa (*e.g.*, Johanneson 1988, Johnson & Black 1984). Little is known about spirorbin dispersal ability, as expected given the general paucity of our knowledge about larval dispersal in the field in general (Todd 1998).

1.3.2 Substrates

Spirorbins commonly settle on many different substrates, including rocks, shells (living or dead), crustacean carapaces, mollusc shells, stalked hyrozoans and bryozoans and thalli of various macrophytes. Some species appear to exclusively prefer hard substrates, whereas others occur exclusively on algae (*e.g.*, Knight-Jones *et al.* 1975) - sometimes even specific algal species (*e.g.*, *Spirorbis corallinae* on coralline algae; de Silva & Knight-Jones 1962; *Pilelaria marginata* on the giant kelp *Macrocystis integrifolia*, pers. obs.) or even a particular region of the blade (on younger regions of *Laminaria* fronds; Stebbing 1972). However, this ecological isolation appears to be unusual, as most species descriptions list various substrate types for a given species (*e.g.*, Knight-Jones & Knight-Jones 1977, Knight-Jones *et al.* 1979, Knight-Jones 1978). For example, *Circeis armoricana* in Barkley Sound, British Columbia live on various algae, from the highly branched *Sargassum muticum* to the thin and leafy *Ulva* spp., as well as living occasionally on rocks, shells, coralline algae and bryozoans (pers. obs.; Knight-Jones *et al.* 1979).

1.3.3 Species interactions

Little is known about the ecological role of spirorbin polychaetes. They may be important food sources for gastropod molluscs, which are their most commonly reported predators (Moran *et al.* 1984, Ward 1989). They are also eaten by small fishes (Knight-Jones *et al.* 1973) and juvenile seastars (Feder 1970). Knight-Jones *et al.* (1975) suggest that rhabdocoel flatworms and mites are also important spirorbin predators due to the high frequency of occurrence in their tubes.

Spirorbin tubeworms have been shown to compete for space with conspecifics (Wisely 1960), but seem to affect the settlement of other animals very little, especially encrusting animals such as bryozoans and sponges, which often overgrow them (Stebbing 1973).

Other biotic interactions are elusive; one exception being the presence of the parasitic isopod *Eisothistos* (= 'invader') in tubes of various spirorbin species. These are described by Knight-Jones & Knight-Jones (unpubl.) but little is known of their biology and effects on the host worm. Spirorbin tubeworms could perhaps be considered parasites themselves; however, the negative effects on their living hosts (whether these be macrophytic algae or invertebrates) remain uninvestigated.

In general, the ecological impact of spirorbin polychaetes within their communities remains unknown. Perhaps they occupy an empty niche, which may contribute to their relative diversity and long-lived presence in the fossil record (Howell 1962).

1.3.4 Fossil record

Polychaete families are often thought to be ancient groups, given the difficulty that arises in inferring relationships among them with both molecular and morphological data (McHugh 2000, Halanych 2002, Rouse & Fauchald 1997, Rouse & Pleijel 2001). The Serpulidae are no exception; they are thought to date back to the Ordovician Period based on the appearance of fossilized calcareous tubes (Ruedemann 1934).

Small, planospirally-coiled calcareous tubes encrust hard substrates aging from the Ordovician to the recent Holocene age (Ruedemann 1934, Benham 1910, Chenu 1843, Ager 1961). These are routinely assigned to the genus *Spirorbis* due to their resemblance to spirorbins. However, recent ultrastructural evidence suggests that these early records (pre-Cretaceous) may not be spirorbins but instead members of the extinct order Microconchida, which are possible lophophorates (Taylor & Vinn 2006). Thus true spirorbins may only date back to the Cretaceous period, and may be a much less ancient group than previously thought.

1.4 SYSTEMATICS OF THE SPIRORBINAE

1.4.1 Relationships among subfamilies of the Serpulidae

The taxonomic history of the Serpulidae dates back to Linnaeus, who described the genus *Serpula*, in the group Vermes Testacea, which contained several serpulids and a few molluscs (Linnaeus 1758). Latreille (1825) recognized the Serpulidae as a family. In 1919, Chamberlain subdivided the Serpulidae into the Spirorbinae and Serpulinae, based on the asymmetrical bodies and coiled tubes of the former. Subsequently, the remaining Serpulinae were subdivided further into the Filograninae (for those serpulids lacking an operculum; Rioja 1923) and Serpulinae (for those with an operculum).

In 1970, Pillai elevated the Spirorbinae to family level ('Spirorbidae') based on their possession of 'several important characters which are peculiar to themselves'. This family status has been called into question, as it renders the remaining Serpulidae paraphyletic (Ten Hove 1984, Fitzhugh 1989). However, the family name 'Spirorbidae' has remained in use by some to accommodate the taxonomic structure erected at the subfamily level (Macdonald 2003, Kupriyanova 2003, Kupriyanova *et al.* 2001, Fauchald 1977). Here, I elect to use the term Spirorbinae in its original form (including all 'spirorbids') as a subfamily within the Serpulidae, to avoid confusion (explained below) and also to reflect their status as a monophyletic group within serpuliform polychaetes, with whom they share important morphological traits.

Relationships among the serpulid subfamilies (Serpulinae, Filograninae, and Spirorbinae) have recently been investigated (Kupriyanova *et al.* 2006, Lehrke *et al.* 2006). The Spirorbinae are thought to be derived within Serpulidae due to their reduction of thoracic chaetigers (Caullery and Mesnil 1897). However, Knight-Jones (1981) hypothesized spirorbin polychaetes were ancestral due to the 'persistence' of the inversion of the fecal groove in serpulids (and sabellids), where it does not seem effective in the absence of a coiled tube. This hypothesis never found much favour, and was rejected by Smith (1991), who found Serpulidae (containing Spirorbinae) to be monophyletic based on morphological data.

Ten Hove (1984) suggested an evolutionary scenario for Serpulidae based on the morphology of the operculum. He suggested that the Filograninae (that often lack an operculum) are the most ancestral spirorbins, and form a paraphyletic grade at the base of the Serpulidae. Serpulinae and Spirorbinae, then, were derived from this grade. This seems appropriate given the similarities in opercular morphology of Serpulinae and Spirorbinae. Both have 'modified' opercular stalks that lack pinnules. Filogranin operculae, if they possess them, are on 'unmodifed' stalks that remain pinnulated like other radioles in the crown.

However, both Kupriyanova *et al.* (2006) and Lerhke *et al.* (2006) reject this hypothesis based on a phylogenetic analysis of morphological and DNA sequence data (28S and 18S rDNA). Sister to Spirorbinae are members of the Filograninae, and these in turn are nested within the Serpulinae. This sheds some new light on evolution in the Serpulidae, as many Filograninae are small-bodied brooders, like the Spirorbinae. Thus we must rethink our hypotheses regarding the evolution of the operculum, and perhaps recognize the importance of a small body size and brooding in the evolution of the Serpulidae.

1.4.2 Relationships within the Spirorbinae

Over 140 described species of spirorbin polychaetes are known worldwide (Kupriyanova *et al.* 2001). These are divided into six tribes (formerly subfamilies) based on their brooding mode (discussed above): Paralaeospirini, Circeini, Spiorbini, Romanchellini, Pileolariini, and Januini. I elect to use tribes to refer to the spirorbin subgroups to accommodate the phylogenetic viewpoint of many authors regarding the lack of family status of Spirorbinae (*e.g.*, Ten Hove 1984, Smith 1991, Rouse & Pleijel 2001) and also to avoid confusion in reference to the use of the name Spirorbinae: it has either been a subfamily within Spirorbidae (containing the only the genus *Spirorbis*), or has referred to the group in its entirety (as I use it here).

Relations among the six spirorbin tribes, and their monophyly, have long been uncertain, and are the focus of Chapters 2 (morphological data) and 3 (molecular data) of this thesis. Traditionally, opercular-brooding is assumed to be the most specialized form of brooding, and is derived within Spirorbinae, as their ancestors were likely tube brooders (Elser 1907, Borg 19a17, Gravier 1923, Potwald 1968, Knight-Jones & Thorp 1984). This assumption is partly based on patterns of speciation among the Spirorbinae: opercular brooders are by far the most speciose (both tribes of opercular brooders encompass more than 70% of described species), thus it appears there must be some benefit to opercular brooding; indeed, it has been viewed as an evolutionary novelty (Macdonald 2003).

A preliminary phylogenetic hypothesis, based on morphological data, supports this hypothesis (Macdonald 2003). However, this topology depended on character weighting, and was constructed at the genus level from species descriptions. Chapter 2 expands on this preliminary analysis with increased taxon sampling at the species level, and a larger number of characters. Chapter 3 further tests this hypothesis using molecular data. These investigations aim to construct a robust phylogenetic hypothesis that will enable us to test evolutionary scenarios about the origins of novel traits in spirorbin polychaetes: body size, brooding mode and body asymmetry.

1.5 NOVEL TRAITS IN THE SPIRORBINAE

Associated with the distinctive morphology of spirorbin polychaetes are novel traits whose evolutionary history stand out as interesting avenues of investigation: miniaturization, unusual and diverse modes of brooding, and conspicuous bilateral asymmetry.

1.5.1 Brooding modes and miniaturization

Miniaturization is often associated with the origin of novelty (Hanken & Wake 1993). Novel structures are newly derived, or permit the assumption of a new function for an existing structure (Mayr 1960). The use of the operculum as a brood chamber fits this definition well. A reconstruction of spirorbin evolutionary history may hold some clues to its origin, and perhaps novel structures in general: do they evolve once within a clade, or multiple times? The dissimilar morphology of the two types of opercular brood chamber certainly suggests the latter is true.

If opercular brood chambers are considered to be 'novel' then tube brooding must be considered ancestral. This seems likely based on their sister-group relationship with members of the Filograninae, of which many are small-bodied tube brooders (none are opercular brooders). Tube brooding has been viewed as less advantageous in light of the diversity of opercular brooders (*e.g.*, Caullery & Mesnil 1897, Borg 1917, Elser 1907, Macdonald 2003). Opercular brooding is considered more advantageous than tube brooding for numerous reasons, including spatial constraints on brood size (Hess 1993), prevention of predation on adult structures (Thorp 1975, Thorp & Segrove 1975) and increased oxygenation, and therefore faster development (and therefore faster turnover) of broods (E. Kupriyanova and P. Knight-Jones pers. comm.).

However, focusing on the benefits of opercular brooding does not give tube brooders their due: perhaps the existence of different types of tube brooding (four of six spirorbin tribes) may be evidence of their 'success'. One point, amidst this conjecture, is clear: we must give our evolutionary hypotheses sufficient phylogenetic support to ensure they are robust. Thus the construction of a robust phylogenetic inference (Chapters 2 and 3) is necessary before we speculate on how the costs and benefits of various brooding modes may have shaped spirorbin evolution (Chapter 4).

1.5.2 Directional asymmetry

The origin of tube coiling in the Spirorbinae is an intriguing phenomenon. It could have many benefits. Daly (1978b) has some interesting suggestions: the spiral form may have enabled the invasion of algal substrates by reducing flexion stress that a meandering tube might incur, it could allow for continual renewal of the main bonding area between the tube and the substrate (around the periphery of the spiral), it may protect the thin-walled inner whorls from damage, or perhaps allow greater population densities by minimizing overlap with neighbours. Coiling remains an interesting puzzle.

Even more puzzling, however, is the fixation of one coiling direction (right or left - dextral or sinistral) over another within a species. Almost all spirorbin tubeworms are directionally asymmetric, meaning that virtually all individuals of the species coil the *same* direction. How this bias towards *a particular side* may have evolved remains a mystery, as it is difficult to imagine why one coiling direction may be favoured over another, especially considering that even artificial selection has not yet generated directional asymmetry in laboratory experiments [with *Drosophila melanogaster; e.g.*, ocelli size (Coyne 1987) and number (Maynard Smith & Sondi 1960); wing folding behaviour (Purnell & Thompson 1973), and the number of thoracic bristles (Tuinstra *et al.* 1990)].

One suggestion is the bias towards a particular side became fixed into the genome by genetic assimilation (Waddington 1953) of an antisymmetric precursor (randomly determined direction of asymmetry with no heritable basis to the directional bias; Palmer 2004). In this way, the phenotype (direction of coiling) would precede the genotype (genes directing the development of asymmetry). This idea is directly at odds with the conventional view of evolution (genotype-precedes-phenotype). The extent to which phenotypic plasticity can result in novel forms is hotly debated (*e.g.,* West-Eberhardt 2003, 2005); perhaps the study of conspicuous bilateral asymmetries may shed some light on these debates, because direction of morphological asymmetry is comparable across many taxa (Palmer 1996a, 1996b, 2004). The possibility of genetic assimilation as a source of novel forms can be tested in a phylogenetic reconstruction of spirorbin evolution: if antisymmetric species are ancestral, then we must accept genetic assimilation as a possible route to directional bias.

The first step to determining their phylogenetic position is to identify antisymmetric species. These are defined as having a random coiling direction (50:50 distribution of left- and right-coiling individuals). One spirorbin species exhibits this random distribution - *Neomicrorbis azoricus* Zibrowius 1972. More species with variable coiling directions exhibit what is termed *situs inversus*, or a rare reversal of coiling in a normally fixed population (*e.g.*, *Paradexiospira vitrea* in the Northeast Pacific, Knight-Jones *et al.* 1979). These species present an opportunity to investigate if coiling direction is genetically determined, or if the environment may influence direction of coiling.

1.6 RESEARCH QUESTIONS AND OVERVIEW OF RESULTS

Current understanding about the evolution of the Spirorbinae is based on non-phylogenetic hypotheses and assumptions about body size evolution (*e.g.*, Knight-Jones *et al.* 1979). Large-bodied spirorbins are presumed to be ancestral, given they tend to have a large number of thoracic segments like non-spirorbin serpulid ancestors. Therefore other traits large-bodied taxa possess (*e.g.*, tube brooding and antisymmetric coiling) are also presumed to be ancestral by default. This thesis sets out to test these assumptions by asking the following questions:

Question 1. What are the relationships among spirorbin tribes?

Based on morphological and sequence data (18s and 28s rDNA), and contrary to previous hypotheses, the Januini are basal and the Pileolariini + Spirorbini occupy the most derived position on the tree. Thus opercular brooding may be ancestral, and tube brooding may have multiple origins.

Question 2. What are the costs and benefits of the various brooding modes?

Benefits of both opercular brooding and tube brooding were apparent. Opercular brooders may be able to start brooding at smaller body sizes, and have the ability to produce large broods at small body sizes (brood size is not tied strongly to body size). Tube brooders, on the other hand, tend to have longer reproductive seasons and tend to produce larger broods at large body sizes (brood size is tied strongly to body size). This may explain the surprising tendency for body size increase observed in primarily tube-brooding clades.

Question 3. Is coiling direction fixed or randomly determined in dimorphic species?

The dimorphic "*Paradexiospira vitrea*" has clearly undergone a speciation event: all evidence points to the genetic and ecological isolation of dextral and sinistral forms. This clearly indicates a genetic basis for coiling direction in this species, and raises questions regarding reports of *situs inversus* in other species.

1.7 SUMMARY

Spirorbin tubeworms provide rare opportunities to study evolutionary transitions among brooding modes and coiling direction. They are a well-defined, monophyletic group of polychaetes that present attractive opportunities to address some broad evolutionary issues. The aim of this thesis is (1) to construct a robust phylogenetic hypothesis with which to test evolutionary hypotheses, and (2) to test assumptions about the evolution of novel traits in the Spirorbinae. In so doing, we may better understand the origins of unique life history characteristics, and potentially shed some light on the prevalence of unconventional evolutionary processes such as genetic assimilation and the origin of novelty.

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CHAPTER 2.

MORPHOLOGICAL PHYLOGENY OF THE SPIRORBINAE (POLYCHAETA, SERPULIDAE) AT THE SPECIES LEVEL*

2.1 INTRODUCTION

At first glance, spirorbin tubeworms (Spirorbinae, Serpulidae) appear morphologically homogeneous: they are all tiny (< 5 mm) filter-feeding polychaetes with distinctive spirally coiled calcareous tubes. However, close inspection reveals a fascinating diversity of form: spirorbins have six different modes of embryonic incubation, and varying degrees of bilateral asymmetry associated with the direction of tube coiling.

These traits are of interest to evolutionary biologists. Both spirorbin brooding modes and coiling asymmetry have arisen in discussions of the origins of novelty (brooding modes: Strathmann *et al.* 1984, Macdonald 2003; asymmetry: Palmer 1996, 2004). Phylogenetic reconstruction of spirorbin evolution may provide clues to fundamental questions about the origin of such novel traits: do they arise only once within a clade, or multiple times? and, how does directionally fixed bilateral asymmetry evolve?

**Expanded from: Macdonald, T.A. 2003. Phylogenetic relations among spirorbid subgenera and the evolution of opercular brooding. Hydrobiologia 496: 125-143.*

2.1.1 Diversity of the Spirorbinae

The family Serpulidae was erected nearly 200 years ago by Latreille (1825). All spirorbins were originally classified in the genus *Spirorbis* (*e.g.*, Caullery and Mesnil 1897, Bush 1904, Pixell 1912) within the Serpulidae. In 1919, Chamberlain established Spirorbinae for those Serpulidae with asymmetric bodies and coiled tubes. All other serpulids were placed in the Serpulinae (marine, with operculae, or modified plug-like radioles), the Filograninae (those lacking an operculum, Rioja 1923) and Ficopomatinae (brackish water dwellers with toothed collar chaetae; Pillai 1960; since removed by Ten Hove & Weerdenburg 1978). The Spirorbinae was elevated to family status (Spirorbidae) by Pillai (1970) due to the possession of 'several important characters peculiar to themselves'.

Subsequently, 'spirorbids' were divided into six subfamilies based on their brooding modes (Bailey 1969; Knight-Jones 1978). The ~130 described species of spirorbins were assigned to 6 subfamilies and 27 genera. However, their family status was debated, as it rendered the remaining Serpulidae (Serpulinae and Filograninae) paraphyletic (*e.g.,* Ten Hove 1984, Fitzhugh 1989). It also led to some confusion: the name "Spirorbinae' became less inclusive, referring to the subfamily containing the genus *Spirorbis*. Given this confusion in terminology, and the well-established placement of spirorbins within Serpulidae (Macdonald 2003, Fitzhugh 1989, Smith 1991), I elect to adopt the original use of 'Spirorbinae' and use tribes to describe taxonomic organization below this level (originally subfamilies; defined by their mode of brooding). This provides the clearest description of their natural groupings and is the easiest scheme to understand in light of their taxonomic history.

2.1.2 Novel brooding modes of the Spirorbinae

The small body size of spirorbins correlates with several life-history traits, including simultaneous hermaphrodism, lecithotrophy and brooding (Kupriyanova *et al.* 2001). The ancestral mode of brooding within the Spirorbinae is thought to be in-tube incubation, which is used by some small serpulids and 4 of the 6 spirorbin tribes (originally subfamilies): the Paralaeospirini, Circeini, Spirorbini, and Romanchellini (Knight-Jones and Fordy, 1979). These 4 tube-incubating subfamilies have distinctive brooding modes: embryos of the Paralaeospirinii are brooded loose in the tube, embryos of the Circeini adhere to the tube and each other by a gelatinous matrix, and embryos of the Spirorbini are attached to the posterior of the tube by a thread to the tube lining. In the Romanchellini, the brood is attached to the adult worm by a thoracic brood-stalk that is thought to be homologous with a reflexed tentacle (Knight-Jones & Thorp 1984).

The remaining two tribes, Januini and Pileolariini, brood their young in a modified plug-like tentacle, or operculum. The opercular brood chambers of these two groups are distinct, both developmentally and morphologically (Knight-Jones & Thorp 1984). Januini have a simple cuticular cup that forms below the distal plate outside of the opercular ampulla. This brood chamber is used only once: larvae are released when the brood chamber dehisces at its base. The base of the brood chamber becomes the distal plate of the next brood chamber. The opercular brood chambers of the Pileolariinii are formed by the invagination of the opercular ampulla, and therefore have walls composed of a double layer of epithelium. These brood chambers have pores at their base, presumably for the entrance and exit of embryos (Thorp 1975, Potswald 1968), and are used for more than one brood. These are more morphologically diverse than the brood chambers of the Januinae, and can resemble either an open cup or a closed ampulla (Kupriyanova *et al.* 2001).

Opercular brood chambers seem to fit Mayr's (1960) definition of an evolutionary novelty, as they appear to be "a newly acquired structure or property that permits the assumption of a new function". Pileolariini and Januini toegether encompass more than two-thirds of described species (Knight-Jones & Fordy 1979), which has led to the conclusion that opercular brooding has spurred a diversification event (Hess, 1993; Macdonald 2003; Macdonald & Rouse *in review*) and therefore has some selective advantage. Possible advantages to opercular brooding are investigated in Chapter 4, a comparison of life history traits among brooding modes.

The above hypothesis assumes homology of the brood chambers of Januini and Pileolariini. However, their morphological distinctiveness may be evidence of their independent origins. Alternatively, opercular brooding could have arisen only once, and the Januini and Pileolariini would be a monophyletic group. The question of whether novelty arises once, or multiple times, in a clade is often debated among evolutionary biologists (*e.g.*, West-Eberhardt 2003, 2005) and is investigated here through phylogenetic reconstruction.

2.1.3 Evolution of directional asymmetry

Spirorbins not only possess unique brooding structures; they are also distinct because of their coiled tubes and correspondingly asymmetric bodies. The evolution of such conspicuous bilateral asymmetries remains a puzzle: for a structure to evolve via natural selection, the phenotypic variation that exists must affect performance (Palmer 1996a). Directional asymmetry (lateral bias or "handedness") seems refractory to selection, as artificial selection cannot produce deviations from bilateral asymmetry in a *particular direction* (Palmer 1996b). So how did it evolve? Palmer (2004) suggests that directional asymmetry has arisen just as many times through the genetic assimilation (Waddington 1953; phenotype-preceding-genotype) of an antisymmetric (with random direction of bias and usually induced by an environmental trigger) precursor as it has through the conventional evolutionary pathway (genotype-preceding-phenotype).

Spirorbins present an opportunity to test whether directional asymmetry may have evolved through an antisymmetric precursor. All spirorbins have coiled tubes, which are either right- or left-coiling (dextral or sinistral). However, one particular species, *Neomicrorbis azoricus* Zibrowius 1972, is antisymmetric. Its large body size and large number of thoracic segments suggests a basal placement of this species (Zibrowius 1972), as do preliminary phylogenetic analyses (Macdonald 2003). However, the phylogeny of Spirorbinae in a rigorous species-level phylogenetic analysis remains untested.

2.1.4 Phylogenetic reconstruction of spirorbin relationships

Testing evolutionary hypotheses such as the origin of novel traits requires thorough species-level phylogenetic reconstruction. This species-level study expands on a previously published genus-level morphological phylogeny (Macdonald 2003) by increasing taxon sampling (including representatives of all tribes and genera; 101 taxa with outgroups) and adding characters. The use of morphological data here allows phylogenetic reconstruction with rare specimens and provides a solid basis for future investigations using molecular data (Chapter 3). Moreover, the consideration of all types of data is important to create robust phylogenetic inferences, and therefore a framework in which to place future studies of life history traits (Chapter 4) and the evolution of asymmetry (Chapter 5).

2.2 METHODS

2.2.1 Taxon Sampling

Of approximately 130 described species of spirorbins (Kupriyanova *et al.* 2001), 90 are represented here (a total 101 taxa with 11 outgroups). Representatives of all tribes and 23 spirorbin genera are included in this analysis (Table 2-1).

Most taxa were studied firsthand from my own collection, and those of P. and E.W. Knight-Jones (University of Wales, Swansea) and G.W. Rouse (South Australia Museum) with few exceptions. Three monotypic genera were unavailable due to rarity of material: *Neomicrorbis azoricus* Rovereto 1904, *Anomalorbis manuatus* Vine 1972 and *Crozetospira dufresnei* Rhzavksy 1997. These species were either from inaccessible locales (abyssal depths in the case of *N. azoricus*; Zibrowius, 1972) and the southern

Indian Ocean in the case of C. *dufresnei* (Rhzavsky, 1997), or are extremely rare (*Anomalorbis manuatus* was described from only a single specimen, Vine 1972). In these cases, data were collected from published species descriptions. Understandably, these three species have uncertain tribal affinities (*Incertae sedis*) due to a lack of reproductive specimens.

Eleven outgroup taxa were included in this analysis. Seven are members of the Serpulidae, both Serpulinae (*Serpula columbiana*, *Crucigera zygophora*, *Pseudochitinopoma occidentalis*, *Galeolaria caespitosa*, *Hydroides norvegicus*) and Filograninae (*Protula* sp. and *Salmacina dysteri*). The sister group of extant spirorbin species is thought to be a member of the Serpulinae, with the Filograninae being the most ancestral serpulid subfamily based on opercular morphology (Ten Hove 1984). Also included were more basal members of the Sabellida, including Sabellidae (*Eudistylia vancouveri* and *Amphiglena terebro*), Sabellaridae (*Sabellaria alveolata*) and Oweniidae (*Owenia fusiformis*).

2.2.2 Morphological characters

Data were limited to those observable using light microscopy, which allowed for wider taxon sampling and fewer missing data. Specimens were mounted in polyvinyl-lactophenol or glyercol for observation of finer details of morphology, such as opercular and chaetal charactertstics. Live or fresh specimens were studied where possible, but many specimens were only available preserved in formalin. Each species was scored for 148 characters (Table 2-2) (gaps treated as missing), which are roughly divided into 5 major categories: developmental and larval characteristics, gross adult morphology, reproductive characteristics, opercular morphology, and morphology of chaetae and uncini. These characters are described in more detail below. Data were compiled using MacClade 4.0 (Maddison & Maddison 2004). Numbers in character descriptions below correspond to character numbers in Table 2-2. The scoring of character states for each species can be found in the character matrix (Table 2-3). Inapplicable data were indicated with '-' and unknown character states were indicated with '?'. These cases were treated as 'missing' in the analysis.

Table 2-1 List of taxa with locality and voucher numbers; K-J refers to the collection of Drs. P. and E.W. Knight-Jones; GWR to that of Greg W. Rouse, and TAM that of Tara A. Macdonald. SAM refers to the South Australia Museum. References included for those taxa not examined in person.

Taxon	Locality	Source
Outgroups		
1 *Owenia fusiformis* (Owenidae)	Barkley Sound, BC	Rouse & Pleijel 2001
2 *Sabellaria alveolata* (Sabellaridae)	Bondi, NSW, Australia	Rouse & Pleijel 2001
3 *Amphiglena terebro* (Sabellidae)	Bondi, NSW, Australia	Rouse & Pleijel 2001
4 *Eudistylia vancouveri* (Sabellidae)	Barkley Sound, BC	SAM E3502
Serpulinae		
5 *Serpula columbiana*	Barkley Sound, BC	SAM E3505
6 *Pseudochitinopoma occidentalis*	Barkley Sound, BC	SAM E3501
7 *Crucigera zygophora*	Barkley Sound, BC	SAM E3503
8 *Galeolaria caespitosa*	Whyalla, South Australia	TAM
9 *Hydroides norvegicus*	Hawaii	Bastida-Zavala 2003
Filograninae		
10 *Protula* sp.	Banyuls, France	GWR
11 *Salmacina dysteri*	Barkley Sound, BC	TAM
Spirorbinae		
Spirorbini		
12 *Spirorbis bidentatus*	La Jolla, CA	K-J
13 *S. bifurcatus*	Barkley Sound, BC	SAM E3489
14 *S. bushi*	Fiji	SAM E3481
15 *S. corallinae*	Finnøy, Norway	SAM E3497
16 *S. cuneatus*	Plymouth, UK	K-J
17 *S. inornatus*	Brittany, UK	K-J
18 *S. marioni*	La Paz, CA	K-J
19 *S. rothlisbergi*	La Jolla, CA	K-J
20 *S. spatulatus*	La Jolla, CA	K-J
21 *S strigatus*	Funchal, Baja California	K-J
22 *S. tridentatus*	Finnøy, Norway	SAM 3477
23 *S. spirorbis*	Sangerdi, Iceland	SAM 3357
24 *S. rupestris*	Finnøy, Norway	SAM 3500
25 *Velorbis gesae*	Madeira	K-J
Circeini		
26 *Circeis armoricana*	Barkley Sound, BC	SAM E3476
27 *C. spirillum*	Stykkishlómør, Iceland	SAM E3507
28 *C. vitreopsis*	Commander Is.	K-J
29 *Paradexiospira violacea*	Cook Inlet, AL	K-J
30 *P. vitrea*	Barkley Sound, BC	SAM E3483
Januini		
31 *Janua pagenstecheri*	Sangerdi, Iceland	SAM E3506
32 *Neodexiospira brasiliensis*	Barkley Sound, BC	SAM E3498
33 *N. fenestrata*	Cape du Coudeic, SA, Aus.	K-J
34 *N. foraminosa*	Ago Bay, Honshu, Japan	K-J

Table 2-1 cont'd

Taxon	Locality	Source
Januini con't		
35 *N. formosa*	Bermuda	K-J
36 *N. kayi*	Inhaca, South Africa	K-J
37 *N. nipponica*	Barkley Sound, BC	SAM E3486
38 *N. pseudocorrugata*	Plymouth, U.K.	K-J
39 *N. steueri*	Encounter Bay, SA, Aus.	SAM E3523
40 *N. turrita*	Hawauu	K-J
41 *Pillaiospira natalensis*	Uvongo, South Natal, Africa	K-J
42 *P. pentaloba*	Red Sea, Saudi Arabia	K-J
43 *P. trifurcata*	Brighton, NSW, Aus.	K-J
44 *Leodora knightjonesi*	West Indies	K-J
Paralaeospirini		
45 *Paralaeospira levinsini*	Cape Town, South Africa	K-J
46 *P. malardi*	Cornwall, UK	K-J
47 *P. monacantha*	Auckland Islands, New Zealand	K-J
48 *P. parallela*	Auckland Islands, New Zealand	K-J
49 *P. sicula*	Borge Bay, South Orkney Islands	K-J
50 *Eulaeospira convexis*	North Bondi, NSW, Aus.	SAM E3496
51 *E. orientalis*	Encounter Bay, SA, Aus.	SAM E3495
Pileolariini		
52 *Pileolaria alata*	Curaçao, West Indies	K-J
53 *P. annectans*	Cape Town, South Africa	K-J
54 *P. berkeleyana*	La Paz, Baja California	K-J
55 *P. daijonesi*	Watamu, Kenya	K-J
56 *P. darkarensis*	SoumbDioun Bay, West Africa	K-J
57 *P. heteropoma*	Plymouth, UK	K-J
58 *P. lateralis*	La Paz, Baja California	K-J
59 *P. marginata*	Barkley Sound, BC	SAM E3478
60 *P. militaris*	Pt. Cartwright, QLD, Aus.	SAM E3492
61 *P. rosepigmentata*	Portsmouth, UK	K-J
62 *P. spinifer*	La Paz, Baja California	K-J
63 *P. tiarata*	San Clemente Island	K-J
64 *P. semimilitaris*	Curaçao, West Indies	K-J
65 *Vinearia koehleri*	Pt. Cartwright, QLC, Aus.	SAM E3475
66 *V. zibrowii*	Grand Recif Madagascar	K-J
67 *Nidificaria dalestraughnae*	Hawaii	K-J
68 *N. dayi*	Oatland Pt., Cape Prov., S. Africa	K-J
69 *N. nidica*	Cape Verde Islands	K-J
70 *N. palliata*	Cape Town, South Africa	K-J
71 *Simplaria pseudomilitaris*	South Australia	K-J
72 *S. potswaldi*	Barkley Sound, BC	SAM E3504
73 *Bushiella abnormis*	Barkley Sound, BC	SAM E3488
74 *B. evoluta*	Kurile Islands, USSR	K-J
75 *B. verruca*	Dodds Narrows, Van. Is, BC	K-J
76 *Jugaria atlantica*	Azores, Mid-Atlantic Ridge	K-J
77 *J. beatlesi*	Kurile Islands, USSR	K-J
78 *J. granulata*	South Wales, UK	K-J
79 *J. quadrangularis*	Barkley Sound, BC	SAM E3479
80 *J. similis*	San Francisco, CA	K-J
81 *Protolaeodora asperata*	Shaw Island, WA, USA	TAM

Table 2-1 cont'd.

Taxon	Locality	Source
Pileolariini con't		
82 *P. uschakovi*	Kurile Islands, USSR	K-J
83 *Amplicaria spiculosa*	Whyalla, SA, Australia	SAM E3490
Romanchellini		
84 *Romanchella bicava*	Amsterdam Is., S. Indian Ocean	K-J
85 *R. pustulata*	Caleta Leandro, Chile	K-J
86 *R. quadricostalis*	Kangaroo Island, SA, Australia	SAM E3491
87 *R. solea*	Poor Knight Islands, New Zealand	K-J
88 *Metalaeospira armiger*	Cape Hallett, Ross Sea	K-J
89 *M. clansmani*	Poor Knights Is., New Zealand	K-J
90 *M. tenuis*	Port Lincoln, SA, Aus.	SAM E3480
91 *Protolaeospira canina*	Cape du Couedic, SA, Aus.	K-J
92 *P. capensis*	Bondi, NSW, Australia	SAM E3484
93 *P. cavata*	Borge Bay, South Orkney Islands	K-J
94 *P. eximia*	Barkley Sound, BC	SAM E3482
95 *P. pedalis*	Signy Island, South Orkney Islands	K-J
96 *P. triflabellis*	Cape du Couedic, SA, Aus.	K-J
97 *Helicosiphon platyspira*	Kerguelen, Prince Edward Islands	K-J
98 *H. bisocensis*	Biscoe Bay, Antarctic Peninsula	K-J
Incertae sedis		
99 *Anomalorbis manuatus*	Hawaii	Vine 1972
100 *Crozetospira dufresnei*	Crozet Islands, Antarctica	Rhzavsky 1997
101 *Neomicrorbis azoricus*	Azores, Mid-Atlantic Ridge	Zibrowius 1972

Table 2-2 List of morphological characters and their character states (see text for details). Non-relevant characters are coded as '-' and missing data as '?' (Gaps are treated as missing in all analyses).

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1 Prostomium: fused to peristomium 0; distinct 1.
2 Shape of prostomium: lobed 0; limited to palps 1.
3 Peristomium: lips only 0; distinct ring 1.
4 Number of peristomial rings: one 1; two 2.
5 Palps: absent 0; present 1.
6 Origin of palps: prostomial 0; peristomial 1.
7 Form of prostomial palps: absent 0; tentacular crown 1.
8 Radiolar lobes: separate 0; dorsally fused 1.
9 Radiolar skeleton: absent 0; present 1.
10 Number of radioles: more than twenty 0; more than ten 1; less than ten 2.
11 Basal fusion of radioles: absent 0; present 1.
12 Nuchal organs: absent 0; present 1.
13 Body divided into thorax and abdomen: absent 0; present 1.
14 Chaetal inversion: absent 0; present 1.
15 Thoracic segment bilateral symmetry: symmetric 0; asymmetric 1.
16 Number of thoracic segments (concave side): > five 0; one 1; two 2; three 3; four 4.
17 Number of thoracic tori (concave side): > five 0; two 2; three 3; four 4.
18 Number of thoracic segments (convex side): >five 0; one 1; two 2; three 3; four 4; five 5.
19 Number of thoracic tori (convex side): >five 0; one 1; two 2; three 3; four 4.
20 Thoracic membrane (collar): absent 0; present 1.
21 Dorsal fusion of collar margin: unfused 0; fused 1.
22 Collar extends into abdominal cloak: absent 0; present 1.
23 Dorsal thoracic crystalline patch: absent 0; present 1.
24 Form of crystalline patch: diffuse 0; single 1; paired 2.
25 Calcareous tube: absent 0; present 1.
26 Coiled tube: absent 0; present 1.
27 Coiling direction: dextral 0; sinistral 1.
28 *Situs inversus* coiling: absent 0; present 1.
29 Stacked whorls: absent 0; present 1.
30 Mouth of tube evolute: absent 0; present 1.
31 Tube type: chalky 0; porcellanous 1; vitreous 2.
32 Tube with longitudinal ridges: absent 0; present 1.
33 Number of longitudinal ridges: one 1; two 2; three 3; four 4; >five 5.
34 Tube with growth rings: absent 0; present 1.
35 Tube with pits in surface: absent 0; present 1.
36 Tube with peripheral flange: absent 0; present 1.
37 Tube with fluted aperture: absent 0; present 1.
38 Maximum coil diameter: 2-5 mm 0; <2 mm 1; > 5 mm 2.
39 Type of larva: planktotrophic 0; lecithotrophic 1.
40 Number of ciliary bands on trochophore larvae: two 2; three 3.
41 Metatrochophore with collar: absent 0; present 1.
42 Larval attachment gland: absent 0; present 1.
43 Number of larval attachment glands: single 1; paired 2.
44 Larval ocelli: absent 0; present 1.
45 Number of pairs of larval ocelli: one 1; two 2; three 3; four 4; five 5.
46 Larvae with anal vesicle: absent 0; present 1.
47 Number of anal vesicles: single 1; paired 2.
48 Development of collar: post-settlement 0; pre-settlement 1.
49 Development of branchial buds: post-settlement 0; pre-settlement 1.
50 Development of operculum: post-settlement 0; pre-settlement 1.
51 Sexuality pattern: gonochorism 0; simultaneous hermophroditism 1.

52 Site of egg production: entire abdomen 0; anterior abdomen 1.
53 Site of sperm production: entire abdomen 0; posterior abdomen 1.
54 Spermatids: single 0; clusters 1; tetrads 2; eights 3.
55 Sperm morphology: spherical head 0; elongate head 1.
56 Embryo incubation: absent 0; present 1.
57 Location of incubation: inside tube 0; inside operculum 1.
58 Tube incubation: embryos loose in tube 0; gelatinous matrix 1; posterior filament 2; thoracic brood-stalk 3.
59 Opercular brood chamber: invaginated epithelial chamber 0; cuticular cylinder 1.
60 Exit of embryos from brood chamber: through pore 0; dehiscence of brood chamber 1.
61 Thoracic brood-stalk morphology: distinct stalk 0; oviduccal funnel (reduced) 1.
62 Origin of brood-stalk: above second thoracic fascicle 0; between second and third thoracic fascicles 1.
63 Maximum brood size: 0-10 0; 10-50 1; 50-100 2; >100 3.
64 Operculum: absent 0; present 1.
65 Number of operculae: single 1; double 2.
66 Opercular stalk pinnules: absent 0; present 1.
67 Operculum location: left (concave) side 0; either side (alternates) 1.
68 Origin of opercular stalk: between first and second radioles 0; outside radioles 1.
69 Opercular stalk long, extending beyond tentacular crown: absent 0; present 1.
70 Operculum with basal processes: absent 0; present 1.
71 Shape of operculum: globular 0; funnel 1; cone 2.
72 Edge of operculum: smooth 0; crenulate 1.
73 Operculum calcified: absent 0; present 1.
74 Location of opercular calcification: distal 0; proximal 1; peripheral 2; plate only 3.
75 Opercular plate: absent 0; present 1.
76 Orientation of opercular plate: perpendicular (even with tube mouth) 0; oblique 1.
77 Opercular plate spines: absent 0; present 1.
78 Primary opercular plate with rim: absent 0; present 1.
79 Secondary opercular plate with rim: absent 0; present 1.
80 Surface of opercular plate: concave 0; convex 1; flat 2.
81 Opercular plate forms cone: absent 0; present 1.
82 More than one opercular plates retained after moulting: absent 0; present 1.
83 Primary opercular plate talon: absent 0; present 1.
84 Secondary opercular plate talon: absent 0; present 1.
85 Location of talon: eccentric 0; peripheral 1.
86 Talon internal or external to opercular epithelia: internal 0; external 1.
87 Shape of talon: vestigial (reduced) 0; tooth-shaped 1; spatulate 2.
88 Talon with wings: absent 0; present 1.
89 Talon with digitate projections: absent 0; present 1.
90 Number of digitate projections: one 1; two 2; three 3; four 4; five 5.
91 Talon with terminal serrations: absent 0; present 1.
92 Talon with depressions: absent 0; present 1.
93 Talon attached to thoracic tori by visible muscle fibre: absent 0; present 1.
94 Fusion of talon and brood chamber: absent 0; present 1.
95 Collar chaetae: absent 0; present 1.
96 Type of collar chaetae: fin and blade 0; simple 1; geniculate 2; bayonet 3.
97 Form of collar chaetae: straightened 0; bent (with joint) 1.
98 Gap between fin and blade: absent 0; present 1.
99 Teeth of collar chaetal blade: fine (cannot distinguish individual teeth) 0; coarse 1.
100 Teeth of collar chaetal fin: fine 0; coarse 1.
101 Collar chaetae with cross-striations: absent 0; present 1.
102 Lateral distribution of cross-striated chaetae: present on both sides 0; absent on concave side 1.
103 Collar chaetae morphology on convex and concave sides: similar 0; different size or shape 1.
104 Capillary collar chaetae: absent 0; present 1.
105 Lateral distribution of capillary collar chaetae: both sides 0; convex side only 1.

106 Margin of chaetae in second thoracic fascicle: smooth 0; serrated 1.
107 Capillary chaetae in second thoracic fascicle: absent 0; present 1.
108 Simple chaetae in third thoracic fascicle: absent 0; present 1.
109 Margins of simple chatae in third thoracic fascicle: smooth 0; serrated 1.
110 Capillary chaetae in third thoracic fascicle: absent 0; present 1.
111 Sickle chaetae in third thoracic fascicle: absent 0; present 1.
112 Shape of sickle chaetae: parallel-sided 0; pennant-shaped 1.
113 Teeth of sickle chaetae: fine 0; coarse 1.
114 Portion of sickle chaetae that is distal (sickle): < 0.25 0; 0.25-0.5 1; >0.5 2.
115 Thoracic uncini: absent 0; present 1.
116 Thoracic uncini with multiple rows of teeth: absent 0; present 1.
117 Number of rows of thoracic uncinal teeth: two 2; three 3; four 4; five 5; six 6; seven 7; eight 8; fifteen 9.
118 Arrangement of rows of thoracic uncinal teeth: straight 0; diagonal 1.
119 Dimensions of thoracic uncinal peg: gouge-shaped 0; flat 1; round 2.
120 Shape of thoracic uncinal peg: square 0; pointed 1; scalloped 2; multi-pronged 3.
121 Number of points in pointed thoracic uncinal peg: one 0; three 1; five 2.
122 Thoracic uncinal peg with lateral teeth: absent 0; present 1.
123 Lateral distribution of thoracic uncini: symmetric 0; more on concave side 1; more on convex 2.
124 Segment with minimum number thoracic uncini: terminal thoracic setiger 0; second convex 2; thirdconvex 3; fourth convex 4.
125 Asetigerous region: absent 0; present 1.
126 Number of abdominal chaetigers: > 30 0; 0-10 1; 11-20 2; 21-30 3.
127 Abdominal chaetae: capillary 0; simple 1; geniculate 2; brush-like 3; trumpet-shaped 4.
128 Shape of geniculate abdominal chaetae: pennant-shaped 0; parallel-sided 1.
129 Geniculate abdominal chaetae with ventral blade ledge: absent 0; present 1.
130 Geniculate abdominal chaetae with projecting heel: absent 0; present 1.
131 Teeth of abdominal chaetae: fine (indistinguishable with light microscope) 0; coarse 1.
132 Size of abdominal chaetae in relation to collar chaetae: same 0; larger 1; smaller 2.
133 Paired abdominal chaetae: absent 0; present 1.
134 Lateral distribution of abdominal chaetae: symmetric 0; asymmetric 1.
135 Anterior-posterior distribution of geniculate abdominal chaetae: entire abdomen 0; posterior 1; anterior 2.
136 Capillary abdominal chaetae: absent 0; present 1.
137 Anterior-posterior distribution of capillary abdominal chaetae: posterior 0; anterior 1; entire abdomen 2; terminal setiger only 3.
138 Lateral distribution of capillary abdominal chaetae: both sides 0; concave side only 1.
139 Abdominal tori on convex side: absent 0; present 1.
140 Anterior-posterior distribution of abdominal tori on convex side: entire abdomen 0; posterior 1; anterior 2.
141 Location of largest abdominal tori (always on concave side): anterior 0; posterior 1; all same size 2; middle 3.
142 Multiple rows of abdominal uncinal teeth: absent 0; present 1.
143 Number of rows of abdominal uncinal teeth: <10 0; >10 1.
144 Arrangement of rows of abdominal uncinal teeth: straight 0; diagonal 1.
145 Dimensions of anterior peg of abdominal uncini: flat 0; gouge-shaped 1.
146 Shape of anterior peg of abdominal uncini: square 0; scalloped 1; flared 2; multi-pronged 3; indented 4; pointed 5.
147 Lateral distribution of abdominal uncini: symmetric 0; asymmetric 1.
148 Abdominal uncini on first abdominal setiger: absent 0; present 1.

(1-7) Prostomium and Peristomium. The prostomium and peristomium are pre-segmental regions in polychaetes that are often modified into sensory and/or feeding structures (Rouse & Pleijel 2001). The prostomium, or first presegmental region, is fused to the peristomium in all taxa excluding *Owenia fusiformis*, which has a well-delineated prostomium. In Oweniidae and Sabellaridae, it is lobed. In the remaining taxa (Sabellariidae, Sabellidae and Serpulidae) it is limited to palps only (2). The peristomium is limited to the mouth region only in Sabellariidae, but in Oweniidae, Sabellidae and Serpulidae it forms a distinct ring (3). These rings are single or paired (4). Also associated with these regions are sensory palps, which are thought to be homologous in all polychaetes (Rouse & Fauchald 1997). They are absent in Oweniidae (5). Palps are either peristomial or prostomial in origin (6) and form the tentacular crown in Sabellidae and Serpulidae (7).

(8-11) Radioles. Radioles can be dorsally fused, as in the Sabellidae, or separate as in the Serpulidae (8). Sabellids also have a radiolar skeleton (9, Fitzhugh & Rouse 1999). Species of Spirorbinae and Filograninae tend to have fewer than ten radioles, whereas the larger-bodied Serpulinae tend to many more than this (10; Ten Hove 1984); however, this character may be correlated with body size. The base of the radioles may be joined together by an interbranchial membrane (11). This membrane is present in some sabellid species, and some Serpulinae (*Serpula, Crucigera*, and *Hydroides*), but is absent in spirorbin species.

(12) Nuchal organs. All polychaetes have nuchal organs, which are a single pair of sensory cilitated structures that are usually external patches or pits (12). In sabellids and serpulids, they have become internalized (Fauchald & Rouse 1997).

(13) Body plan. The Sabellidae and Serpulidae have distinct thoracic and abdominal regions (*e.g.*, Fauchald 1977; 13). Associated with this body plan is chaetal inversion (14), the reversal of the dorsoventral arrangement of chaetae and uncini in the thorax and abdomen. In the thorax, uncini are ventral (neurochaetae) and become dorsal (notochaetae) in the abdomen. This situation is clear-cut in the Sabellidae and Serpulidae only; Fitzhugh (1989) suggests that chaetal inversion may be present in sabellariids. However, it is not present in the Oweniidae, which lack distinct body regions.

(15-19) Thoracic segments. The bodies of Oweniidae, Sabelllidae, and non-spirorbin members of Serpulidae are symmetrical, distinguishing them from the Spirorbinae, which possess body asymmetry corresponding with tube coiling direction. This asymmetry is most apparent in the thoracic segments (15). In the Spirorbinae, the number of thoracic segments and tori on the convex and concave sides of the body are important taxonomic characters (*e.g.*, Knight-Jones *et al.*, 1979) (16-19).

(20-22) Thoracic membrane. The thoracic membrane is a synapomorphy of the Serpulidae (Fauchald & Rouse 1997; 20). In the Spirorbinae, it can be fused on the ventral surface (21), and often is extended into an 'abdominal cloak' (22), which covers

part of the abdomen and is thought to have a role in the transfer of embryos to reproductive structures (Potswald 1967,1968).

(23-24) Crystalline patch. Many spirorbins possess a granular pigmented area on their dorsal surface (*e.g.*, Knight-Jones *et al.* 1979; 23). They can be paired, single or diffuse (24).

(25-38) Tube morphology. The calcareous tube is a synapomorphy of the Serpulidae (Rouse & Pleijel 2001; 25). Calcareous tubes are not present in the outgroup taxa, whose tubes are constructed with sand grains or mud. Tube coiling is characteristic of the Spirorbinae (26). The direction of this coil has historically been an important taxonomic character (27) and is often consistent among members of a tribe. In some species, there are records of *situs inversus*, or a rare reversal in coiling direction in a normally fixed population (28). The coil can be flat, with all whorls attached to the substrate, stacked (29) or evolute (30), which are often characteristic of aggregating species. Three major types of tubes are apparent: chalky (thick, dull and hard), porcellanous (thin and brittle), or vitreous (glassy and hard) (31). Most serpulids have chalky tubes. Tubes can have various types of sculpturing such as longitudinal ridges, growth rings or surface pits (32-35), and may have peripheral flanges or fluted aperatures (36-38). The Spirorbinae have a uniform maximum coil diameter within species (38).

(39-47) Larval traits. Serpulidae have both planktotrophic and lecithotrophic larvae (39; Kupriyanova *et al.* 2006). All spirorbin larvae are lecithotrophic. Their

trochophores have a distinct number of ciliary bands (40) and their metatrochophores possess a collar (41). Later in development, some spirorbin larvae develop what are termed 'attachment glands', which are easily visible in settling larvae and, if present, can be single or paired (42-43). The other readily apparent larval characteristics are ocelli (44), which are lacking only in the Oweniidae. They are always paired, but the number of pairs varies within the Serpulidae (45). Serpulid and sabellid larvae possess anal vesicles (46), which are single or paired (47).

(48-50) Developmental traits. The developmental timing of the thoracic membrane (or collar; 48), branchial buds (49; these give rise to the branchiae, or tentacular crown), and operculum (50) varies in the Serpulidae in relation to the timing of larval settlement (Höglund 1951).

(51) Sexuality pattern. Serpulids are either gonochoristic (separate sexes; this may be confused with sequential hermaphrodism) or simultaneous hermaphrodites, as in the Spirorbinae (Ten Hove 1984).

(52-53) Site of gamete production. Eggs and sperm are produced in different regions of the abdomen in spirorbins (eggs in the anterior segments and sperm in the posterior segments, Potswald 1968), but are produced in the whole abdomen in gonochoristic taxa (*Owenia, Sabellaria,* sabellids and non-spirorbin serpulids).

(54-55) Sperm characteristics. Sperm are released individually in *Owenia, Sabellaria*, and non-spirorbin serpulids. In spirorbins, they are released in clusters, tetrads or eights (54). The heads of spirorbin sperm are elongate (and spherical in other taxa; 55), a characteristic often associated with brooding.

(56-64) Embryo incubation. Brooding (56) is characteristic of all spirorbins, and some filogranins. Few of the Serpulinae brood; most are broadcast spawners, like the sabellids included here, *Sabellaria* and *Owenia*. Spirorbins either brood inside their tube or inside a modified operculum (57).

(58) Tube incubation. Tube-brooding Spirorbinae have various embryo-attachment structures. These have traditionally been used to delineate tribes within the Spirorbinae (originally subfamilies under Spirorbidae; Bailey 1969).

(59-60) Opercular brood chambers. Currently we recognize two types of opercular brood chamber, distinguished both by their structure and development (59; Knight-Jones & Thorp 1984). Larvae are release either through a pore or dehiscence of the chamber (60).

(61-62) Brood-stalk. Romanchellini have a thoracic brood-stalk, which may be reduced to an 'oviduccal funnel' (61; Knight-Jones & Knight-Jones 1994). The origin of this stalk in relation to thoracic segments varies among genera (62).

(63) Maximum brood size. Although brood size is related to body size and embryo size (Chapter 3), it appears to provide reliable phylogenetic information because maximum brood sizes are consistent within species.

(64-74) Operculum. The operculum is a modified plug-like tentacle that is characteristic of Serpulidae, although is lacking in some Filograninae (64). It is single in the Spirorbinae, but some Serpulinae possess two (65). It is borne on a stalk with or without pinnules (66). It is always on the concave side of the worm in spirorbins, but can be found on either side in the remaining serpulids (67). The stalk usually originates between the second and third radioles, but this position varies among members of the Spirorbinae (68). Some Spirorbinae have a conspicuously long opercular stalk that extends far beyond the tentacular crown, and unusual trait among the Serpulidae (69). In some Serpulinae, the opercular stalk has basal processes (70). The operculum itself varies in shape among subfamilies of the Serpulidae (71). It can have either a smooth or crenulate lip (72). In the Spirorbinae, operculae are calcified (73) and the form and placement of this calcification can be a useful taxonomic character (Knight-Jones & Thorp 1984; 74).

(75-82) Opercular plate. Spirorbinae have a calcified opercular plate (75) and its morphology can be diagnostic. Its orientation (76), armourment (77-79) and surface shape (80-81) are often used as taxonomic characters (*e.g.*, Knight-Jones *et al.* 1979, Knight-Jones & Knight-Jones 1977). In some species, more than one opercular plate is retained upon formation of a new one, giving the operculum a stacked appearance (82).

(83-94) Talon. Often associated with the opercular plate is the talon, or ventral projection (83). Secondary talons (those formed with replacement operculae) can be lacking (84). The primary talon can be eccentric or peripheral on the opercular plate (85), and is internal (within the operculum) or external (outside opercular epithelium) (86). They have distinctive shapes (87), and can possess wing-like (88) or finger-like projections (89-90), serrations (91) and depressions (92). These characteristics can be diagnostic when delineating species (Thorp 1975; Knight-Jones & Thorp 1984). The talon can be connected to the thoracic tori by a muscular fibre (93), or fused to the brood chamber in some Pileolariini (94).

(95-103) Collar chaetae. Serpulidae have distinctive chaetae in their first thoracic segment, termed collar chaetae, that are absent in sabellids and other outgroups (95). This fascicle of chaetae is not associated with a torus, or uncini-bearing reduced parapodium. They vary in morphology (96-97), and often possess fin and blade portions. There may be a gap between the fin and blade (98), and either region may have coarse or fine serrations (99-100). Collar chaetae may possess cross-striations (101), and this may differ on the convex and concave sides in the asymmetric Spirorbinae (102). The collar chaetae themselves may differ in morphology on the convex and concave sides as well (103).

(104-105) Capillary collar chaetae. Capillary chaetae may accompany collar chaetae in the thoracic fascicles (104). These may be present on both sides of the body in spirorbins, or may occur on only the concave and convex sides (105).

(106-107) Chaetae of second thoracic segment. All Spirorbinae have simple limbate chaetae in their second thoracic segment. These may have smooth or serrated margins (106) and may be associated with capillary chaetae (107).

(108-114) Chaetae of remaining thoracic segments. Chaetae in the third thoracic segment of the Spirorbinae are similar to those of all remaining thoracic segments (if more than three segments exist). There may be simple limbate chaetae, as in segment 2 (108), which may have smooth or serrated margins (109). Associated with these chaetae may be capillary chaetae (110) or sickle chaetae (111). Sickle chaetae shape (112), serration (113), and blade proportion (114) are useful taxonomic characters (Knight-Jones & Fordy 1979).

(115-124) Thoracic uncini. The Sabellidae and Serpulidae all possess thoracic uncini which are reduced comb-like chaetae common to many tube-dwelling polychaetes. These are lacking in the Sabellariidae and Oweniidae (115). They may have multiple rows of teeth (116), the number of which can be a useful taxonomic character among the Spirorbinae (117). Multiple rows of teeth can be arranged diagonally or straight across the uncinus (118). Each uncinus has an anterior peg, which can be either flat or gouge-shaped (119), and square or pointed (120). In those uncini with a pointed peg, the number of points can be diagnostic at the species level (Knight-Jones *et al.* 1975; 121), and can be associated with lateral teeth (122). The distribution of thoracic tori can be extremely asymmetrical in some Spirorbinae, but not markedly so in others and serpulids and sabellids (123). The torus with the minimum number of uncini is not always the

terminal thoracic setiger, but can be more anterior; and this distribution appears to be consistent within species (124).

(125) Achaetigerous region. The Spirorbinae have a unique region of their bodies between the thorax and the abdomen.. It does not have any chaetae and is thought to develop from a single segment (Knight-Jones 1981). It is often longer than the thorax itself.

(126) Number of abdominal chaetigers. The average spirorbin has much fewer abdominal segments than outgroups; almost always they have less than 30, whereas in some serpulids and sabellids these segments can number in the hundreds.

(127-133) Morphology of abdominal chaetae. The morphology of spirorbin abdominal chaetae (127) are distinctive; they are knee-like (geniculate) and are either pennant-shaped or parallel-sided (128). These may have a lateral 'ledge' formed from uneven chitinous shafts of the chaeta (129), and may have a projecting heel (130). The blade can be coarsely serrated (131). Abdominal chaetae are often much smaller than collar chaetae, but in some taxa can be the same size or larger (132).

(133-135) Distribution of abdominal chaetae. Abdominal chaetae often occur singly, but can be paired (133) and may be distributed asymmetrically on the concave and convex sides of the body (134), often absent entirely or rare on the convex side (especially in tube brooders). The anterior-posterior distribution also varies; more

chaetae are usually found in the posterior fascicles of the abdomen (135) instead of occurring fairly regularly throughout the whole abdomen as in the outgroups included here.

(136-138) Capillary abdominal chaetae. Capillary chaetae are often associated with abdominal chaetae (136). In spirorbins, these are often more numerous posteriorly (137). They may exhibit lateral asymmetry, found only on the concave side of the body as in *Protolaeospira* and some Circeini and Paralaeospirini (138).

(139-141) Abdominal tori. All taxa included here have abdominal tori (uncini-bearing segments; *O. fusiformis* has neuropodial tori for their entire length). In the Spirorbinae, the convex side of the body may be lacking tori, as in some Circeini, Paralaeospirini, and Romanchellini (139). In spirorbins with convex abdominal tori, these may be found only posteriorly instead of throughout the entire abdomen (140). The location of the largest abdominal tori may be anterior, posterior, or located more medially in the abdomen (141).

(142-148) Abdominal uncini. All taxa included here have abdominal uncini (*O. fusiformis* has uncini-like neuropodial hooks). These may have multiple transverse rows of teeth, varying in number (142-143). These rows may be arranged diagonally or straight across (144). As with the thoracic uncini, the anterior peg end may be flat or gouge-shaped (145), and may be square or pointed (146). The distribution of uncini varies between concave and convex sides in some spirorbins (147-148).

Table 2-3 Character matrix. Taxon numbers (left) refer to those associated with species names in Table 2-1. Character numbers (top) refer to characters listed in Table 2-2. Non-applicable characters are coded with '-' and unknown character states with '?'. Gaps are treated as missing in analyses.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1	1	0	1	1	0	-	-	-	-	2	0	0	0	0	0	2	2	2	2	0	-	-	0	-	0	0	-	-	-	-	-	0	-	0	-	-	-	-	0	-
2	0	0	0	-	1	1	0	-	-	0	0	1	1	1	0	0	0	0	0	0	-	-	0	-	0	0	-	-	-	-	-	0	-	0	-	-	-	-	0	3
3	0	1	1	2	1	0	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	-	0	0	-	-	-	-	-	0	-	0	-	-	-	-	0	3
4	0	1	1	2	1	0	1	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	-	0	0	-	-	-	-	-	0	-	0	-	-	-	-	0	3
5	0	1	1	2	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	0	-	1	0	-	-	0	0	0	0	-	0	0	-	0	-	0	3
6	0	1	1	2	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	-	1	0	-	-	0	0	0	1	1	0	0	1	0	-	0	3
7	0	1	1	2	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	1	0	-	1	0	-	-	0	0	0	0	-	0	0	0	0	-	0	3
8	0	1	1	2	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	-	1	0	-	-	0	0	0	0	-	0	0	0	0	-	0	3
9	0	1	1	2	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	0	-	1	0	-	-	0	0	0	0	-	0	0	0	0	-	0	3
10	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	0	0	0	0	1	0	0	0	-	1	0	-	-	0	0	0	1	1	0	0	0	0	-	1	3
11	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	0	0	0	1	1	0	0	0	-	1	0	-	-	0	1	0	0	-	0	0	0	0	-	0	3
12	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	1	0	0	0	1	1	3	0	0	1	0	1	1	2
13	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	0	1	0	0	1	1	2	0	0	1	0	1	1	2
14	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	1	0	0	0	1	0	-	0	0	0	0	1	1	2
15	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	1	0	1	0	1	0	-	0	0	0	0	1	1	2
16	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	1	0	0	0	1	1	3	0	0	1	0	1	1	2
17	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	1	0	-	1	1	1	0	1	0	1	0	-	1	0	1	0	0	1	2
18	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	0	0	0	0	1	1	2	0	0	0	0	1	1	2
19	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	0	1	0	0	1	0	-	0	0	0	0	1	1	2
20	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	0	1	0	0	1	1	1	0	0	0	0	1	1	2
21	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	1	0	0	0	1	1	2	0	0	0	0	1	1	2
22	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	1	0	1	0	1	1	3	0	0	0	1	0	1	2
23	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	1	0	-	1	1	1	0	0	0	1	0	-	0	0	1	0	0	1	2
24	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	1	0	0	1	1	0	-	1	0	0	0	0	1	2
25	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	1	-	1	1	1	0	1	0	1	1	2	1	0	1	0	1	1	2
26	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	0	1	1	0	1	0	0	1	1	3	1	0	0	0	1	1	2
·27	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	0	1	1	0	0	0	1	1	0	-	1	0	0	0	0	1	2
28	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	0	1	1	1	0	0	0	2	1	1	0	0	0	0	0	1	2
29	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	0	1	0	1	1	0	0	1	0	2	1	3	0	0	0	0	0	1	2
30	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	1	1	0	1	1	0	1	1	0	2	1	3	1	0	0	0	1	1	2
31	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	0	0	0	1	0	1	3	1	0	1	0	1	1	2
32	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	0	-	1	1	0	0	1	0	0	1	3	0	0	0	0	1	1	2
33	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	0	-	1	1	0	0	0	1	0	1	3	1	1	1	0	1	1	2
34	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	0	-	1	1	0	0	0	1	0	1	3	0	1	0	1	1	1	2
35	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	0	-	1	1	0	0	1	0	0	1	3	1	0	0	0	1	1	2
36	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	0	-	1	1	1	1	0	0	0	1	1	1	0	0	0	1	1	2
37	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	0	-	1	1	0	0	0	0	0	1	3	1	1	0	0	1	1	2
38	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	0	-	1	1	0	0	0	0	0	1	3	0	0	0	0	1	1	2
39	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	0	-	1	1	0	1	1	0	0	1	3	1	1	0	0	1	1	2
40	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	0	-	1	1	0	0	1	0	0	1	3	0	1	1	0	0	1	2
41	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	0	0	1	0	1	1	3	1	0	0	1	1	1	2
42	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	0	0	0	0	0	1	3	1	0	0	0	1	1	2
43	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	0	0	1	0	0	1	3	0	0	0	0	1	1	2
44	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	1	0	0	1	0	1	3	1	0	1	0	1	1	2
45	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	0	0	-	1	1	1	0	0	1	1	0	-	1	0	0	1	0	1	2
46	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	0	0	-	1	1	1	0	1	0	1	1	1	0	0	0	0	1	1	2
47	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	1	0	-	1	1	1	0	0	0	0	0	-	0	0	0	0	1	1	2
48	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	0	0	-	1	1	1	0	0	0	0	0	-	0	0	0	0	1	1	2
49	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	0	0	-	1	1	1	0	0	1	1	1	2	0	0	1	0	1	1	2
50	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	1	0	0	0	1	0	-	1	0	1	0	0	1	2
51	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	0	-	1	1	1	0	0	0	1	0	-	1	0	1	0	1	1	2

Table 2-3 cont'd.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
52	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	0	-	1	0	0	1	1	1	2
53	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	1	1	1	1	0	0	1	0	0	1	1	0	0	1	1	1	2
54	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	2	0	0	0	1	1	1	2
55	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	?	1	1	1	0	0	1	0	1	2	1	0	0	1	1	1	2
56	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	2	1	0	1	1	1	1	2
57	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	3	0	0	0	0	1	1	2
58	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	1	1	1	1	0	0	1	0	0	-	1	0	0	1	1	1	2
59	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	1	1	1	1	0	0	1	0	0	-	1	0	0	1	0	1	2
60	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	1	1	1	1	0	0	1	0	0	-	1	0	0	1	0	1	2
61	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	2
62	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	1	1	1	1	0	0	0	0	0	-	1	0	0	1	1	1	2
63	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	1	1	1	1	0	0	0	0	1	3	0	0	0	1	1	1	2
64	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	1	1	1	1	0	0	0	0	1	3	1	0	0	0	0	1	2
65	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	3	1	0	1	1	1	1	2
66	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	3	0	0	0	0	1	1	2
67	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	3	0	0	0	0	1	1	2
68	0	1	1	2	1	0	1	0	0	1	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	1	0	0	-	1	0	0	0	0	1	2
69	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	2	1	0	1	0	1	1	2
70	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	1	1	2	1	1	1	0	0	1	0	0	-	0	0	1	0	1	1	2
71	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	0	-	0	0	0	1	1	1	2
72	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	1	1	2	1	1	1	0	0	1	0	0	-	1	0	0	1	0	1	2
73	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	3	0	0	1	1	0	1	2
74	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	1	0	0	-	0	0	0	1	0	1	2
75	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	1	0	0	0	1	0	1	2
76	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	1	0	0	0	1	1	1	2
77	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	1	0	0	1	0	1	1	2
78	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	1	1	2	1	1	1	0	0	0	0	0	-	0	0	0	0	1	1	2
79	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	2	0	0	1	0	0	1	2
80	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	2	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	2
81	0	1	1	2	1	0	1	0	0	1	0	1	1	1	0	3	2	3	2	1	0	1	1	2	1	1	1	0	0	1	0	1	1	0	0	0	0	0	1	2
82	0	1	1	2	1	0	1	0	0	2	1	1	1	1	0	3	2	3	2	1	0	1	1	2	1	1	1	0	0	1	0	0	-	0	0	0	0	0	1	2
83	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	4	5	3	1	0	0	0	-	1	1	1	0	0	0	0	1	2	0	0	0	0	0	1	2
84	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	1	0	1	1	0	0	0	0	0	1	3	0	0	1	0	1	1	2
85	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	1	0	1	1	1	0	0	0	0	1	3	1	0	1	0	0	1	2
86	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	1	0	1	1	1	0	0	1	1	1	3	0	0	0	0	1	1	2
87	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	1	0	1	0	1	1	1	0	0	0	1	0	-	0	0	1	0	0	1	2
88	0	1	1	2	1	0	1	0	0	1	0	1	1	1	1	4	3	3	2	1	0	0	0	-	1	1	1	0	1	0	1	1	3	0	0	1	0	0	1	2
89	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	0	0	-	1	1	1	0	1	0	1	0	-	0	0	0	0	0	1	2
90	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	0	0	-	1	1	1	0	0	1	1	1	1	1	0	0	0	0	1	2
91	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	0	0	-	1	1	1	0	0	1	1	1	2	1	0	1	1	0	1	2
92	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	0	0	-	1	1	1	0	0	0	1	0	1	1	0	0	1	0	1	2
93	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	3	2	1	0	0	0	-	1	1	1	0	1	0	1	1	1	0	0	1	0	0	1	2
94	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	4	3	1	0	0	0	-	1	1	1	0	0	0	1	0	-	1	0	0	1	0	1	2
95	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	4	3	1	0	0	0	-	1	1	1	0	0	0	1	1	3	0	0	0	0	0	1	2
96	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	4	3	4	3	1	0	0	?	?	1	1	1	0	0	0	1	1	1	1	0	0	1	0	1	2
97	0	1	1	2	1	0	1	0	0	1	0	1	1	1	1	4	3	3	2	1	0	0	?	?	1	1	1	0	0	1	0	0	-	0	0	0	0	0	1	2
98	0	1	1	2	1	0	1	0	0	1	0	1	1	1	1	4	3	3	2	1	0	0	?	?	1	1	1	0	0	1	0	0	-	0	0	0	1	0	1	2
99	0	1	1	2	1	0	1	0	0	2	0	1	1	1	1	5	4	4	3	1	0	0	0	-	1	1	0	?	0	0	0	1	3	0	0	0	0	0	1	2
100	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	3	2	3	2	1	0	0	1	0	1	1	1	0	0	0	2	0	-	0	0	0	0	0	1	2
101	0	1	1	2	1	0	1	0	0	2	0	1	1	1	0	0	0	0	0	1	0	0	1	0	1	1	1	1	0	0	2	1	5	0	1	0	0	2	1	2

Table 2-3 cont'd.

	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
1	-	0	-	0	-	0	-	-	-	-	0	0	0	0	0	0	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	0	0	-	1	1	0	-	-	-	-	0	0	0	0	0	0	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	0	0	-	1	1	1	2	-	0	-	0	0	0	0	0	0	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	0	0	-	1	2	1	2	-	0	-	0	0	0	0	0	0	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	0	0	-	1	2	1	1	0	0	1	0	0	0	0	0	0	-	-	-	-	-	-	-	1	2	0	1	0	0	1	1	1	0	-	0	-	-	-	-	-
6	0	0	-	1	2	1	1	0	0	1	0	0	0	0	0	0	-	-	-	-	-	-	-	1	1	0	1	0	1	0	0	0	0	-	0	-	-	-	-	-
7	0	0	-	1	2	1	1	0	0	1	0	0	0	0	0	0	-	-	-	-	-	-	-	1	2	0	1	0	0	1	1	1	0	-	0	-	-	-	-	-
8	0	0	-	1	2	1	2	0	0	1	0	0	0	0	0	0	-	-	-	-	-	-	-	1	1	0	1	0	0	0	0	0	0	-	0	-	-	-	-	-
9	0	0	-	1	2	1	1	0	0	1	0	0	0	0	0	0	-	-	-	-	-	-	-	1	2	0	1	0	0	0	1	1	0	-	0	-	-	-	-	-
10	0	0	-	1	2	1	2	0	0	1	0	0	0	0	1	0	-	-	-	-	-	-	-	1	1	1	1	0	0	0	0	0	0	-	0	-	-	-	-	-
11	0	0	-	1	2	1	2	0	0	-	0	0	0	0	0	1	0	0	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-
12	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	1
13	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
14	1	1	1	1	2	1	2	1	1	0	1	1	1	2	1	1	0	2	-	-	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	1	0	1	1	2
15	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	2
16	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	0
17	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	2
18	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	2
19	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	2	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
20	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
21	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	2
22	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	2
23	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	2
24	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	0	2	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	0
25	1	1	1	1	2	1	2	1	1	1	1	1	1	1	1	1	0	2	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	0
26	1	0	-	1	3	1	2	1	1	0	1	1	1	1	1	1	0	1	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
27	1	0	-	1	3	1	2	1	1	0	1	1	1	1	1	1	0	1	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	3	1	0	0	0	0	0
28	1	0	-	1	3	1	2	1	1	0	1	1	1	1	1	1	0	1	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	2
29	1	0	-	1	3	1	2	1	1	0	1	1	1	1	1	1	0	1	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
30	1	0	-	1	3	1	2	1	1	0	1	1	1	1	1	1	0	1	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
31	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	1	1	1	0	0	0	0	0	2	0	1	3	1	0	0	0	0	2
32	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
33	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	1	1	2
34	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	1	1	0
35	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	1	1	2
36	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	1	1	1
37	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	1	2
38	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	1	1
39	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	1	1	0
40	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	2
41	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	1	1
42	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	0	1	1	0	0	0	0	0	2	0	1	2	1	0	0	1	1	0
43	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	0	1	1	0	0	0	0	0	2	0	1	2	1	0	0	0	1	1
44	1	1	2	1	3	1	2	1	1	0	1	1	1	1	1	1	1	-	1	1	-	-	0	1	1	0	0	0	0	0	2	0	1	2	1	0	0	0	0	2
45	1	0	-	1	2	1	2	1	1	0	1	1	1	2	1	1	0	0	-	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	1	0	0	2
46	1	0	-	1	2	1	2	1	1	0	1	1	1	2	1	1	0	0	-	-	-	-	1	1	1	0	0	0	1	0	2	0	1	0	1	0	0	0	0	2
47	1	0	-	1	2	1	2	1	1	0	1	1	1	2	1	1	0	0	-	-	-	-	1	1	1	0	0	0	1	0	2	0	1	2	1	0	1	1	1	2
48	1	0	-	1	2	1	2	1	1	0	1	1	1	2	1	1	0	0	-	-	-	-	1	1	1	0	0	0	1	0	2	0	1	0	1	1	0	0	0	2
49	1	0	-	1	2	1	2	1	1	0	1	1	1	2	1	1	0	0	-	-	-	-	1	1	1	0	0	0	1	0	2	0	1	0	1	1	0	0	0	1
50	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	0	-	-	-	-	1	1	1	0	0	0	1	0	2	0	1	0	1	0	0	1	1	2
51	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	0	-	-	-	-	1	1	1	0	0	0	1	0	2	0	1	0	1	0	0	1	0	2

Table 2-3 cont'd.

	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
52	1	1	1	1	2	1	2	1	1	0	1	1	1	2	1	1	1	-	0	-	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	1	1	1	0
53	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	1	0	0	2	0	1	0	1	0	1	0	0	1
54	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
55	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
56	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	1	1	0
57	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
58	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	1	0	0
59	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	1	0	0
60	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	1	0	1	0
61	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
62	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
63	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	1	1	0	2
64	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	1	1	0	1
65	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	0	0	2	0	1	1	1	0	0	1	1	0
66	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	1	0	2	0	1	1	1	0	0	0	0	2
67	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	1	0	2	0	1	1	1	0	0	0	0	2
68	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	2	1	1	0	0	0	0	0	2	0	1	1	1	0	0	0	0	0
69	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	0	0	2	0	1	1	1	0	0	0	0	0
70	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	1	1	0	0	0	0	0
71	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	1	1	0	0
72	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	1	1	1	0
73	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
74	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	2	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	2
75	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	2	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	2
76	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	1	0	2	0	1	0	1	0	0	0	0	2
77	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	2	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	1
78	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	0	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	1	1
79	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	1
80	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	1	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	2
81	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	3	1	1	0	0	1	0	0	2	0	1	0	1	0	0	0	0	0
82	1	1	1	1	2	1	2	1	1	0	1	1	1	1	1	1	1	-	0	0	-	-	3	1	1	0	0	1	0	0	2	0	1	0	1	0	0	0	0	0
83	1	?	?	1	?	1	2	1	1	0	1	1	1	2	1	1	1	-	0	1	-	-	0	1	1	0	0	0	0	0	2	0	1	1	1	0	1	1	1	2
84	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	0	1	3	1	1	0	0	0	1	0	2	0	1	0	1	1	0	0	0	2
85	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	0	1	3	1	1	0	0	0	1	0	2	0	1	0	1	1	0	0	0	0
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87	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	1	1	2	1	1	0	0	0	1	0	2	0	1	0	1	1	0	0	0	2
88	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	0	-	-	-	-	1	1	1	0	0	0	1	0	2	0	1	2	1	0	0	1	1	2
89	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	0	-	-	-	-	1	1	1	0	0	0	1	0	2	0	1	2	1	0	0	0	0	0
90	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	0	-	-	-	-	1	1	1	0	0	0	1	0	2	0	1	0	1	0	0	1	0	0
91	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	0	0	2	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
92	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	0	0	3	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	1
93	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	0	0	2	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	1
94	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	0	0	3	1	1	0	0	0	0	0	2	0	1	0	1	1	0	1	1	0
95	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	1	0	2	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	2
96	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	0	0	2	1	1	0	0	0	0	0	2	0	1	0	1	1	0	0	0	0
97	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	0	0	3	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	0
98	1	0	-	1	2	1	2	1	1	0	1	1	1	3	1	1	0	3	-	-	0	0	3	1	1	0	0	0	0	0	2	0	1	0	1	0	0	0	0	2
99	1	?	?	1	?	1	2	1	1	0	1	1	1	?	1	1	0	?	-	-	?	?	?	1	1	0	0	0	0	0	2	0	1	0	1	0	0	1	1	0
100	1	?	?	1	3	1	2	1	1	0	1	1	1	?	1	1	0	?	-	-	?	?	?	1	1	0	0	0	1	0	2	0	1	0	1	1	0	0	0	1
101	1	?	?	1	?	1	2	1	1	0	1	1	1	?	1	1	?	?	?	-	-	-	?	1	1	0	0	0	0	0	2	0	1	1	1	0	0	0	0	0

Table 2-3 cont'd.

	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0	0	-	0	0	-	-	-	-	0	0
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0	0	1	0	0	0	-	-	-	0	0
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0	0	1	0	0	0	-	-	-	1	1
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	-	-	0	0	1	0	0	0	-	-	-	1	1
5	0	-	0	-	-	-	-	-	-	-	-	-	-	-	1	3	0	1	0	1	0	2	0	0	-	0	0	1	0	0	0	-	-	-	1	0
6	0	-	0	-	-	-	-	-	-	-	-	-	-	-	1	0	0	1	0	?	0	2	0	0	-	0	0	1	0	0	0	-	-	-	1	0
7	0	-	0	-	-	-	-	-	-	-	-	-	-	-	1	3	0	1	0	1	0	2	0	0	-	0	0	1	0	0	0	-	-	-	1	0
8	0	-	0	-	-	-	-	-	-	-	-	-	-	-	1	3	0	1	0	-	0	2	0	0	-	0	0	1	0	0	0	-	-	-	1	0
9	0	-	0	-	-	-	-	-	-	-	-	-	-	-	1	0	0	1	0	1	0	2	0	0	-	0	0	1	0	0	0	-	-	-	1	0
10	0	-	0	-	-	-	-	-	-	-	-	-	-	-	1	1	0	-	0	-	0	2	0	0	-	0	0	1	0	0	0	-	-	-	1	0
11	0	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0	0	1	0	0	0	2	0	0	-	0	0	1	0	0	0	-	-	-	1	0
12	0	0	1	1	0	0	1	0	1	1	0	0	0	-	1	0	1	1	0	1	0	2	0	1	0	0	0	1	0	0	1	1	1	2	1	1
13	0	0	1	1	0	0	1	0	1	1	0	0	0	-	1	0	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	0	1	2	1	1
14	0	0	1	1	0	0	?	0	1	1	0	0	0	-	1	0	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	0	1	2	1	1
15	0	0	1	1	1	0	2	0	0	-	0	0	0	-	1	0	1	1	0	1	0	2	0	1	0	0	1	1	0	1	1	1	0	2	1	1
16	0	0	1	1	0	0	2	1	0	-	0	0	0	-	1	0	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	0	0	1	1	1
17	0	0	1	1	1	0	2	1	0	-	0	0	0	-	1	0	1	1	0	1	1	1	0	0	-	0	0	1	0	0	1	1	0	2	1	1
18	0	0	1	1	0	0	1	0	1	3	0	0	0	-	1	0	1	1	0	1	1	0	0	0	-	0	1	1	0	1	1	0	1	2	1	1
19	0	0	1	1	0	0	1	0	0	-	0	0	0	-	1	0	1	1	0	1	1	0	0	1	0	0	1	1	0	1	1	0	1	2	1	1
20	0	0	1	1	0	0	2	0	0	-	0	0	0	-	1	0	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	0	1	2	1	1
21	0	0	1	1	0	0	1	0	0	-	0	0	0	-	1	0	1	1	0	1	1	0	0	1	0	0	1	1	0	1	1	0	1	1	1	1
22	0	0	1	1	0	0	1	0	0	-	0	0	0	-	1	0	1	1	0	1	0	2	1	1	0	0	1	1	0	1	1	0	1	1	1	1
23	0	0	0	0	-	-	0	-	-	-	-	-	0	-	1	0	1	1	0	1	0	2	0	1	0	0	1	1	0	1	1	0	1	1	1	1
24	0	0	0	0	-	-	0	-	-	-	-	-	0	-	1	0	1	1	0	1	0	2	0	1	0	0	1	1	0	1	1	0	1	1	1	1
25	0	0	0	0	-	-	0	-	-	-	-	-	0	-	1	0	1	1	0	1	0	2	0	1	0	0	0	1	0	0	1	0	1	1	1	1
26	0	0	1	1	0	0	2	0	0	-	0	0	1	-	1	2	1	-	0	-	0	2	0	1	0	0	1	1	0	1	0	-	-	-	1	1
27	0	0	1	1	0	0	2	0	0	-	0	0	0	-	1	2	1	-	1	-	1	1	1	1	0	0	1	1	0	1	0	-	-	-	1	1
28	0	0	1	1	1	0	2	0	0	-	1	0	0	-	1	2	1	-	0	-	0	2	0	1	0	0	0	1	0	0	0	-	-	-	1	1
29	0	0	1	1	1	0	1	0	0	-	0	0	0	-	1	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	1	0	1	0	1	1
30	0	0	0	0	-	-	-	-	-	-	-	-	1	-	1	0	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	1	1	1
31	0	0	1	1	1	0	0	0	0	-	0	0	0	0	1	1	1	-	0	-	0	1	1	1	0	0	0	1	0	1	1	0	0	2	1	1
32	0	0	1	1	1	0	2	0	0	-	1	0	0	0	1	1	1	-	0	-	1	1	1	1	0	0	0	1	0	0	0	-	-	-	1	1
33	0	0	1	1	1	0	2	0	0	-	0	0	0	0	1	1	1	-	1	-	1	1	1	1	0	0	0	1	0	0	0	-	-	-	1	1
34	0	0	1	1	1	0	2	1	0	-	0	0	0	0	1	1	1	-	0	-	1	1	1	1	0	0	0	1	0	0	0	-	-	-	1	1
35	0	0	1	0	1	0	2	1	0	-	0	0	0	0	1	1	1	-	0	-	0	2	1	1	0	0	0	1	0	0	0	-	-	-	1	1
36	0	0	1	0	1	0	2	1	0	-	0	0	0	0	1	1	1	-	0	-	0	1	1	0	-	0	0	1	0	0	0	-	-	-	1	1
37	0	0	1	0	1	0	2	0	0	-	0	0	0	0	1	1	1	-	0	-	0	1	1	1	0	0	0	1	0	0	0	-	-	-	1	1
38	0	0	1	0	1	0	2	0	0	-	0	0	0	0	1	1	1	-	1	-	1	1	1	1	0	0	1	1	0	1	0	-	-	-	1	1
39	0	0	1	0	0	0	2	1	0	-	0	0	0	0	1	1	1	-	1	-	1	1	1	1	0	0	0	1	0	0	0	-	-	-	1	1
40	0	0	1	0	0	0	2	0	0	-	0	0	0	0	1	1	1	-	1	-	1	1	1	1	0	0	0	1	0	0	0	-	-	-	1	1
41	0	0	1	0	1	0	0	0	0	-	0	0	0	0	1	1	1	-	1	-	1	1	1	1	0	0	0	1	0	0	0	-	-	-	1	1
42	0	0	1	0	1	0	1	0	1	5	0	0	0	0	1	1	1	-	0	-	0	2	1	1	0	0	0	1	0	0	0	-	-	-	1	1
43	0	1	1	0	1	0	2	0	0	-	0	0	0	0	1	1	1	-	0	-	0	2	1	1	0	0	0	1	0	0	0	-	-	-	1	1
44	0	1	1	1	1	0	1	0	0	-	0	0	0	0	1	1	1	-	1	-	0	2	1	1	0	0	1	1	0	1	0	-	-	-	1	1
45	0	0	1	1	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	0	0	-	0	1	0	1	0	1	1	0	1	0	1	2	1	1
46	0	0	1	1	0	0	1	0	0	-	0	0	0	0	1	0	1	1	0	1	0	-	0	1	0	0	1	1	0	1	1	0	1	2	1	1
47	0	0	0	0	-	-	-	-	-	-	-	-	0	-	1	0	1	1	1	1	0	-	0	1	1	1	0	1	1	0	1	0	1	2	1	1
48	0	0	0	0	-	-	-	-	-	-	-	-	0	-	1	0	1	1	1	1	0	-	0	1	0	1.	0	1	1	0	1	1	1	2	1	1
49	1	0	1	0	0	0	1	0	0	-	0	0	0	-	1	0	1	1	0	0	0	-	0	1	0	0	0	1	0	0	1	0	1	2	1	1
50	?	0	0	0	0	0	-	0	0	-	0	0	0	-	1	0	1	1	?	?	0	2	0	0	-	0	0	1	0	0	0	-	-	-	1	1
51	0	0	0	0	-	-	-	-	-	-	-	-	0	-	1	0	1	1	0	1	0	2	0	0	-	0	0	1	0	0	0	-	-	-	1	1

Table 2-3 cont'd.

	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116
52	0	0	1	0	0	0	2	1	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	1	1	2	1	0
53	0	0	0	0	-	-	-	-	-	-	-	-	1	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	1	1	2	1	0
54	0	0	1	0	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	1	0	1	1	0
55	0	0	1	0	0	0	0	0	0	-	0	0	0	0	1	0	0	1	1	1	1	0	0	1	0	0	0	1	0	0	1	1	1	2	1	0
56	0	0	1	0	0	0	1	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	1	1	2	1	1
57	0	0	1	0	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	2	1	0	0	1	0	0	0	1	0	0	1	1	0	2	1	1
58	0	0	1	0	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	0	1	2	1	0
59	0	0	1	0	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	0	1	1	1	0
60	0	0	1	0	0	0	2	1	1	1	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	1	1	2	1	1
61	0	0	1	0	0	0	1	0	0	-	0	0	0	0	1	0	1	1	1	2	1	0	0	1	0	0	0	1	0	0	1	0	1	2	1	1
62	0	0	1	0	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	0	1	1	1	0
63	0	0	1	0	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	1	1	1	1	0
64	0	0	1	0	0	0	?	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	0	1	2	1	1
65	0	1	1	1	1	0	2	1	0	-	0	0	0	0	1	0	1	0	0	2	0	2	0	1	0	0	1	1	0	1	1	1	1	2	1	1
66	0	1	1	1	1	0	0	0	0	-	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	1	1	0	1	1	0	1	2	1	1
67	0	0	1	1	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	1	1	0	0	0	1	0	0	1	1	0	2	1	1
68	0	0	1	1	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	1	0	2	1	1
69	0	0	1	1	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	1	0	2	1	0
70	0	0	1	1	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	0	0	1	1	0
71	0	0	1	1	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	?	0	0	0	1	0	0	0	-	-	-	1	0
72	0	0	1	1	0	0	0	0	0	-	0	0	0	0	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	0	-	-	-	1	0
73	0	1	1	0	1	1	2	0	0	-	0	0	0	0	1	1	1	-	0	-	0	0	0	?	0	0	0	1	0	0	1	1	1	2	1	1
74	0	1	1	0	1	1	2	0	0	-	0	0	0	1	1	1	1	-	0	-	0	0	0	1	0	0	0	1	0	0	1	1	1	2	1	1
75	0	1	1	0	1	1	2	1	0	-	0	0	0	1	1	1	1	-	0	-	0	0	0	?	0	0	0	1	0	0	1	1	1	2	1	1
76	0	1	1	0	1	1	2	1	0	-	0	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	0	1	0	0	1	1	1	1	1	1
77	0	1	1	0	1	1	2	1	0	-	0	0	0	0	1	0	1	1	0	1	0	0	0	0	-	0	0	1	0	0	1	1	1	1	1	1
78	0	1	1	0	1	1	2	1	0	-	0	0	0	0	1	0	1	1	0	0&1	0	0	0	1	0	0	0	1	0	0	1	1	1	2	1	1
79	0	1	1	0	1	1	2	1	0	-	0	0	1	0	1	0	1	1	0	0	0	0	0	1	0	0	0	1	0	0	1	1	1	2	1	1
80	0	1	1	0	1	1	2	1	0	-	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	1	0	0	1	1	1	2	1	1
81	0	1	1	1	1	0	2	0	0	-	0	0	0	0	1	1	0	-	0	-	0	0	1	1	0	0	0	1	0	0	1	1	1	2	1	1
82	0	1	1	1	1	0	2	0	0	-	0	0	1	0	1	1	0	-	0	-	0	0	1	1	0	0	0	1	0	0	1	0	1	1	1	1
83	0	1	1	1	1	1	0	0	0	-	0	0	0	-	1	0	1	1	1	1	0	2	0	0	-	0	0	1	0	0	1	0	1	2	1	1
84	0	1	1	1	0	0	1	0	0	-	0	1	0	-	1	1	0	-	0	-	0	-	1	1	0	0	0	1	0	0	1	0	1	2	1	1
85	0	1	1	1	0	0	1	0	0	-	0	1	1	-	1	1	0	-	0	-	0	-	1	1	0	0	0	1	0	0	1	0	1	1	1	1
86	0	1	1	1	0	0	1	0	0	-	0	0	0	-	1	1	0	-	0	-	0	-	1	1	0	0	0	1	0	0	1	0	1	1	1	1
87	0	1	1	1	0	0	1	0	0	-	0	0	0	-	1	1	0	-	?	-	0	-	1	1	1	0	0	1	0	0	1	0	1	1	1	1
88	0	0	1	1	0	0	2	0	0	-	0	0	0	-	1	1	1	-	0	-	0	2	0	1	0	0	0	1	0	0	1	0	1	2	1	1
89	0	0	0	0	-	-	-	0	-	-	-	-	0	-	1	1	1	-	0	-	0	2	0	1	1	0	0	1	0	0	1	0	1	1	1	1
90	0	0	0	1	-	-	-	0	-	-	-	-	0	-	1	1	1	-	0	-	0	2	1	1	0	0	0	1	0	0	1	0	1	1	1	1
91	0	0	1	1	0	0	1	0	0	-	0	0	0	-	1	0	1	1	1	1	1	0	1	1	0	0	0	1	0	0	1	0	1	1	1	0
92	0	0	1	1	0	0	1	0	0	-	1	0	0	-	1	0	1	1	1	0	1	0	1	1	0	0	1	1	0	1	1	0	1	1	1	0
93	0	0	1	1	0	0	1	0	0	-	0	0	0	-	1	0	1	1	0	0	0	2	1	1	0	0	1	1	0	1	1	0	1	2	1	0
94	0	0	1	1	0	0	1	0	1	1	1	0	1	-	1	0	1	1	1	1	1	0	0	1	0	0	0	1	0	0	1	0	1	1	1	0
95	0	0	1	1	0	0	1	0	0	-	1	0	0	-	1	0	1	1	0	0	0	2	0	1	0	0	1	1	0	1	1	0	1	1	1	1
96	0	0	1	1	0	0	1	0	1	3	1	0	0	-	1	0	1	1	-	0	1	0	0	1	0	0	1	1	0	1	1	0	1	0	1	0
97	0	1	1	1	1	0	1	0	0	0	0	0	1	-	1	1	1	-	0	-	1	0	0	1	0	0	1	1	0	1	0	-	-	-	1	1
98	0	1	1	1	0	0	2	0	0	0	1	0	0	-	1	1	1	-	0	-	1	0	0	1	0	0	1	1	0	1	0	-	-	-	1	1
99	0	0	1	0	0	0	2	1	0	-	0	0	0	-	1	1	1	-	0	-	0	-	0	1	0	0	1	1	0	1	0	-	-	-	1	1
100	1	0	0	0	-	-	-	-	-	-	-	-	0	-	1	2	1	-	1	-	1	0	0	1	0	0	1	1	0	1	1	0	1	1	1	1
101	0	0	1	1	1	0	2	1	0	-	0	0	0	-	1	0	1	0	0	0	0	2	0	1	0	0	0	1	0	1	0	-	-	-	1	1

Table 2-3 cont'd.

	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148
1	-	-	-	-	-	-	-	0	0	0	0	-	-	-	-	-	1	0	-	1	2	0	1	0	0	1	0	-	-	-	0	1
2	-	0	1	0	-	0	0	0	0	0	1	-	0	0	0	2	1	0	-	1	0	0	1	0	0	1	0	0	0	0	0	1
3	6	0	1	0	-	0	0	0	0	0	1	-	0	0	0	2	1	0	-	1	0	0	1	0	0	1	0	0	0	0	0	1
4	6	0	1	0	-	0	0	0	0	0	1	-	0	0	0	2	1	0	-	1	0	0	1	0	0	1	0	0	0	0	0	1
5	-	0	1	0	-	0	0	0	0	0	4	-	0	0	0	2	1	0	-	1	0	0	1	0	0	1	0	0	0	0	0	1
6	-	0	1	0	-	0	0	0	0	0	2	0	0	0	0	2	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1
7	-	0	1	0	-	0	0	0	0	0	4	-	0	1	1	2	1	0	-	1	0	0	1	0	0	1	0	0	0	0	0	1
8	-	0	1	0	-	0	0	0	0	0	4	-	0	0	0	2	1	0	-	1	0	0	1	0	0	1	0	0	0	0	0	1
9	-	0	1	0	-	0	0	0	0	0	1	-	0	0	0	2	1	0	-	1	0	0	1	0	0	1	0	0	0	0	0	1
10	-	0	1	0	-	0	0	0	0	0	2	0	0	0	0	2	1	0	0	0	-	0	1	0	0	1	0	0	0	0	0	1
11	-	0	1	0	-	0	0	0	0	0	2	0	0	0	0	0	1	0	0	0	-	0	1	0	0	1	0	0	0	0	0	1
12	4	1	1	0	-	0	2	0	1	1	2	0	0	1	1	2	0	0	0	1	0	0	1	0	0	0	-	-	0	0	0	1
13	4	1	0	0	-	0	2	0	1	3	2	0	0	1	0	2	1	0	0	1	0	0	1	0	0	0	-	-	0	0	0	1
14	4	1	0	0	-	?	2	0	1	3	2	0	0	1	1	2	?	0	0	1	0	0	1	0	3	0	-	-	0	0	0	1
15	4	1	0	0	-	0	2	0	1	2	2	0	0	1	1	2	0	0	0	1	0	0	1	0	0	0	-	-	0	0	0	1
16	4	0	0	0	-	0	2	0	1	2	2	0	0	0	0	2	0	0	0	1	0	0	1	0	3	0	-	-	0	0	0	1
17	4	1	0	0	-	0	0	0	1	3	2	0	0	1	1	2	0	0	0	1	0	0	1	0	0	0	-	-	0	0	0	1
18	3	1	0	0	-	0	2	0	1	3	2	0	0	1	1	2	?	0	0	1	0	0	1	0	0	0	-	-	0	0	0	1
19	4	1	0	0	-	0	2	0	1	2	2	0	0	1	1	1	1	0	0	1	0	0	1	0	0	0	-	-	0	0	0	1
20	4	1	0	0	-	0	2	2	1	2	2	0	0	1	1	2	1	0	0	1	0	0	1	0	0	0	-	-	0	0	0	1
21	4	0	0	0	-	0	2	2	1	2	2	0	0	1	1	2	1	0	0	1	0	0	1	0	1	0	-	-	0	0	0	1
22	4	1	0	0	-	0	2	2	1	2	2	0	0	1	1	0	1	0	0	1	0	0	1	0	0	0	-	-	0	0	0	1
23	4	1	1	0	-	0	2	0	1	3	2	0	0	0	1	2	1	0	0	1	0	0	1	0	0	0	-	-	0	0	0	1
24	4	1	1	0	-	0	2	2	1	2	2	0	0	1	1	2	1	0	0	1	0	0	1	0	0	0	-	-	0	0	0	1
25	3	1	1	0	-	0	2	2	1	2	2	0	0	0	1	2	1	0	0	0	-	-	1	0	0	1	1	1	0	0	1	1
26	6	0	1	0	-	0	1	2	1	2	2	0	0	1	1	2	1	0	0	0	-	0	0	-	0	1	1	0	0	0	1	0
27	6	0	1	0	-	0	1	2	1	2	2	0	0	1	1	2	1	0	0	0	-	0	0	-	0	1	1	0	0	0	1	0
28	6	0	1	0	-	0	1	2	1	2	2	0	0	1	1	0	1	0	0	0	-	0	0	-	0	1	1	0	0	0	1	0
29	6	0	1	0	-	0	1	2	1	2	2	0	0	1	1	2	0	0	0	0	-	0	0	-	0	1	1	0	0	0	1	0
30	6	0	1	0	-	0	1	2	1	2	2	0	0	1	0	2	0	0	0	0	-	0	0	-	0	1	1	0	0	0	1	0
31	6	1	-	1	0	1	1	2	1	1	2	1	0	1	0	1	0	0	0	0	-	0	1	0	0	1	0	1	0	1	0	0
32	5	1	-	1	0	1	0	2	1	1	2	1	0	0	1	1	0	0	0	0	-	0	1	0	0	1	0	1	0	2	0	1
33	5	1	-	1	0	1	0	2	1	1	2	1	0	0	1	1	0	0	0	0	-	0	1	0	0	1	1	1	0	2	0	1
34	6	1	-	1	0	1	0	2	1	1	2	1	0	0	0	1	1	0	0	0	-	0	1	0	0	1	0	1	0	2	0	1
35	5	1	-	1	0	1	0	2	1	1	2	1	0	0	1	1	1	0	0	0	-	0	1	0	0	1	0	1	0	2	0	1
36	6	1	-	1	0	1	0	2	1	1	2	1	0	0	1	1	0	0	0	0	-	0	1	0	0	1	0	1	0	2	0	1
37	6	1	-	1	1	1	0	2	1	1	2	1	0	0	1	1	0	0	0	0	-	0	1	0	0	1	0	1	0	2	0	1
38	5	1	-	1	0	1	0	2	1	1	2	1	0	0	1	1	0	0	0	0	-	0	1	0	0	1	0	1	0	1	0	1
39	5	1	-	1	0	1	0	2	1	1	2	1	0	0	1	1	0	0	0	0	-	0	1	0	0	1	0	1	0	1	0	1
40	3	1	-	1	0	1	1	2	1	1	2	1	0	1	1	1	0	0	0	0	-	0	1	0	0	1	0	1	0	2	0	1
41	8	1	-	1	0	1	1	2	1	2	2	1	0	1	1	0	1	0	0	0	-	0	1	0	0	1	0	1	0	2	0	1
42	7	1	-	1	1	1	1	2	1	2	2	1	0	0	1	2	0	0	0	0	-	0	1	0	0	1	0	1	0	2	0	1
43	4	1	-	1	1	1	1	2	1	2	2	1	0	0	1	1	0	0	0	0	-	0	1	0	0	1	1	1	0	2	0	1
44	6	1	-	1	0	1	1	2	1	1	2	0	0	1	1	2	1	0	0	0	-	0	1	0	0	1	1	1	0	0	0	1
45	2	1	1	0	-	0	1	2	1	2	2	0	0	1	1	2	1	1	0	1	0	0	0	-	0	1	1	1	0	4	1	0
46	2	1	1	0	-	0	1	2	1	2	2	0	1	1	1	2	1	1	0	1	-	0	0	-	0	1	0	1	0	1	1	0
47	2	1	0	0	-	0	1	2	1	2	2	0	0	1	1	2	1	1	0	1	0	0	0	-	0	1	1	1	0	1	1	0
48	3	1	1	0	-	0	1	2	1	2	2	0	0	1	1	2	1	1	0	1	0	0	0	-	0	1	0	1	0	0	1	0
49	2	1	1	0	-	0	1	2	1	2	2	0	1	1	1	2	1	0	0	0	-	0	0	-	0	1	0	1	0	0	1	0
50	3	1	1	1	0	0	1	0	1	2	0	-	-	-	-	2	1	0	0	1	2	0	1	1	0	1	0	1	0	3	1	0
51	3	1	1	1	0	0	1	0	1	1	0	-	-	-	-	2	1	0	0	1	2	0	1	1	0	1	0	1	0	3	1	0

Table 2-3 cont'd.

	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148
52	-	-	0	0	-	0	1	2	1	2	2	0	0	1	0	2	1	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
53	-	-	0	0	-	0	0	2	1	2	2	0	0	0	1	2	1	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
54	-	-	0	0	-	0	1	2	1	1	2	0	0	1	1	2	0	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
55	-	-	0	0	-	0	0	2	1	2	2	0	0	1	1	2	1	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
56	2	1	0	0	-	0	1	2	1	2	2	0	0	1	0	2	0	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
57	2	1	0	0	-	0	0	2	1	2	2	0	0	1	1	2	0	0	1	0	-	0	1	0	1	0	-	1	1	0	0	1
58	-	-	0	0	-	0	1	2	1	2	2	0	0	1	1	2	0	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
59	-	-	0	0	-	0	1	2	1	1	2	0	0	1	1	0	0	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
60	3	1	0	0	-	0	0	2	1	2	2	0	0	1	1	2	0	0	1	0	-	0	1	0	1	0	-	1	1	0	0	1
61	3	1	0	0	-	0	0	2	1	2	2	0	0	1	1	2	0	0	1	0	-	0	1	0	1	0	-	1	1	0	0	1
62	-	-	0	0	-	0	1	2	1	2	2	0	0	1	1	2	0	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
63	-	-	0	0	-	0	1	2	1	2	2	0	0	1	1	2	0	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
64	2	1	0	0	-	0	1	2	1	1	2	0	0	1	1	2	0	0	1	1	0	0	1	0	1	0	-	1	0	0	0	1
65	2	1	0	0	-	0	1	0	1	2	2	0	0	1	1	2	1	0	0	0	-	0	1	0	1	0	-	1	0	4	0	1
66	6	1	0	0	-	0	1	0	1	1	2	0	0	1	1	2	0	0	0	0	-	0	1	0	1	0	-	1	0	0	0	1
67	3	1	0	0	-	0	1	0	1	1	2	0	0	1	1	2	0	0	0	1	0	0	1	0	0	0	-	1	0	0	0	1
68	4	1	0	0	-	0	0	0	1	3	2	0	0	1	0	2	0	0	0	1	0	0	1	0	0	0	-	1	0	0	0	1
69	-	-	0	0	-	0	1	0	1	2	2	0	0	0	0	2	0	0	0	0	-	0	1	0	0	0	-	1	0	0	0	1
70	-	-	0	0	-	0	1	0	1	2	2	0	0	1	1	2	1	0	0	1	0	0	1	0	0	0	-	1	0	0	0	1
71	-	-	0	0	-	0	1	2	1	1	2	0	0	1	1	2	1	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
72	-	-	0	0	-	0	1	2	1	3	2	0	0	1	1	2	1	0	1	1	0	0	1	0	1	0	-	1	1	0	0	1
73	3	1	0	0	-	0	1	0	1	3	2	0	0	1	1	2	0	0	0	1	2	0	1	0	0	0	-	1	0	0	0	1
74	2	1	0	0	-	0	1	0	1	3	2	0	0	0	1	2	0	0	0	1	2	0	1	0	0	0	-	1	0	0	0	1
75	2	1	0	0	-	0	1	0	1	3	2	0	0	1	1	2	0	0	0	1	2	0	1	0	0	0	-	1	0	0	0	1
76	2	1	0	0	-	0	1	0	1	2	2	0	0	0	0	2	1	0	0	1	2	0	1	0	0	0	-	1	0	0	0	1
77	2	1	0	0	-	0	1	0	1	2	2	0	0	1	1	0	1	0	0	1	2	0	1	0	0	0	-	1	0	0	0	1
78	2	1	0	0	-	0	1	0	1	2	2	0	0	0	1	2	1	0	0	0	-	0	1	0	1	0	-	1	0	0	0	1
79	3	1	0	0	-	0	1	0	1	2	2	0	0	1	1	2	1	0	0	1	0	0	1	0	0	0	-	1	0	0	0	1
80	2	1	0	0	-	0	1	0	1	2	2	0	0	1	1	2	1	0	0	1	2	0	1	0	0	0	-	1	0	0	0	1
81	2	1	0	0	-	0	1	0	1	2	2	0	0	1	1	2	1	0	0	1	2	0	1	0	0	0	-	1	0	0	0	1
82	2	1	0	0	-	0	1	0	1	2	2	0	0	0	1	0	0	0	0	1	2	0	1	0	0	0	-	1	0	0	0	0
83	2	1	0	0	-	0	1	4	1	2	2	0	0	1	1	2	1	0	2	0	-	-	1	0	3	1	0	1	0	0	0	0
84	4	1	0	0	-	0	1	2	1	2	3	0	0	1	1	2	1	0	0	1	2	0	1	1	0	1	0	1	0	0	1	0
85	4	1	0	0	-	0	1	2	1	3	3	0	0	1	1	2	1	0	0	1	0	0	1	1	0	1	0	1	0	0	1	0
86	9	1	0	0	-	0	1	2	1	2	3	0	0	1	1	2	1	0	0	1	0	0	1	1	0	1	1	1	0	0	1	0
87	9	1	0	0	-	0	1	2	1	2	3	0	0	1	1	2	0	0	0	1	0	0	1	1	0	1	0	1	0	0	1	0
88	2	1	0	0	-	0	1	0	1	2	3	-	0	1	1	2	1	0	0	0	-	0	1	1	2	1	0	1	0	4	1	0
89	2	1	1	0	-	0	1	0	1	2	3	-	0	1	?	2	1	1	0	1	0	0	1	1	2	1	0	1	0	4	1	0
90	4	1	2	0	-	0	1	0	1	2	3	-	0	1	1	2	1	1	0	1	0	0	1	1	2	1	0	1	0	0	1	0
91	-	-	0	0	-	0	1	0	1	2	3	-	0	1	1	2	1	1	0	1	2	1	0	1	0	1	0	1	0	0	1	0
92	-	-	0	0	-	0	1	0	1	2	3	-	0	1	1	2	1	1	0	1	2	1	0	1	0	1	0	1	0	0	1	0
93	-	-	0	0	-	0	1	0	1	2	3	-	0	1	1	2	1	1	0	1	2	1	0	1	0	1	0	1	0	0	1	0
94	-	-	0	0	-	0	1	0	1	3	3	-	0	1	1	2	1	1	0	0	-	1	0	1	0	1	1	1	0	0	1	0
95	2	1	0	0	-	0	1	3	1	2	3	-	1	1	1	2	1	1	0	0	-	1	0	1	0	1	0	1	0	0	1	0
96	-	-	0	0	-	0	1	0	1	0	3	-	0	1	1	2	1	1	0	1	2	1	0	1	0	1	1	1	0	0	1	0
97	2	1	0	0	-	0	1	0	1	0	3	-	0	0	1	0	1	1	0	1	0	1	1	1	0	1	0	1	0	0	1	0
98	2	1	0	0	-	0	1	0	1	0	3	-	0	0	1	0	1	1	0	1	0	1	1	1	0	1	0	1	0	0	1	0
99	6	1	-	1	0	1	1	2	1	1	2	1	0	0	1	2	0	0	0	1	0	0	1	1	3	1	0	1	0	5	1	0
100	4	0	1	0	-	0	?	?	1	1	2	0	0	1	1	0	0	0	0	-	-	-	0	-	0	1	1	0	0	0	1	0
101	9	0	1	0	-	0	0	3	1	0	2	0	0	1	1	2	1	0	0	0	-	-	1	0	0	1	1	0	0	0	0	1

2.2.3 *Phylogenetic analysis*

The resulting matrix had 101 taxa (including 11 outgroups, 7 Serpulidae, 2 Sabellidae, and one member each of the Sabellariidae and Oweniidae) and 148 characters (137 were parsimony-informative). There were 116 binary characters and 32 multi-state. The most parsimonious trees were found using PAUP* 4.0 v.b10 (Swofford 2003; Heuristic searches, tree-bisection reconnection (TBR) branch-swapping, random addition sequence, with 10 trees held at each step. Branch support was obtained using bootstrapping (1000 replicates, heuristics searches, TBR, simple addition sequence) and decay analysis (Bremer 1994; implemented in Autodecay 4.0 (Ericksson 1998)). Decay values were rescaled in the weighted analyses to make them comparable to those of the unweighted analyses (Bremer 1994).

Three different weighting schemes were used. The first had all characters of equal weight. The second was run with characters weighted based on their rescaled consistency index (RCI) and a base weight of 1000. This weighting scheme can correct for differential influence of binary and non-binary characters (Farris 1989) given that multi-state characters tend to contribute more to the total tree length (having a larger number of possible steps), but have lower RCIs. However, if there are a large number of binary characters (as in this study), this 'correction' may not have influence. Nevertheless, the aim here is to assess the sensitivity of inferred topologies to different weighting schemes and not necessarily the broad questions of the value certain weighting schemes over others.

The third weighting scheme was perhaps more arbitrary but relies on knowledge of taxonomists, as it weights all traditionally important taxonomic characters more heavily. For example, previous non-phylogenetic hypotheses of spirorbin relationships have relied on the following characters to discern relationships among genera and tribes (mostly based on Knight-Jones & Fordy 1979): number of thoracic segments and tori (characters 16-19), presence and fusion of the thoracic membrane (20 and 21), larval attachment glands (P. Knight-Jones pers. comm.; characters 42 and 43), spermatid arrangement (G. Rouse, pers. comm.; character 54), brooding mode (Bailey 1969; characters 57, 58, 59), type of collar chaetae (96), presence of sickle chaetae in third thoracic fascicles (112), arrangement of uncinal teeth rows (118 for thoracic,144 for abdomen) and type of abdominal chaetae (127). Therefore, these 16 characters are all given a weight of 5. This scheme downweights those characters believed to be more plastic, such as tube characteristics (Rhzavsky 1994), secondary chaetal characters (Rouse & Fauchald 1997) and operculum ornamentation (Beckwitt 1981), and characters describing the degree of body asymmetry (which may be a convergent characteristic of tube brooders; Vine 1972). A weight of 5 was chosen to reflect the relative importance of these characters in the taxonomy of Spirorbinae. However, various weighting schemes used (*e.g.*, 2 instead of 5) gave similar results but the larger weight amplified these differences.

The evolution of novel traits, including opercular brooding and directional asymmetry, was reconstructed using ancestral-state reconstruction package implemented in Mesquite (Maddison & Maddison 2004) with maximum parsimony methods.

2.3 RESULTS

2.3.1 Support for inferred topologies

Overall, many clades lacked statistical support. Most basal relationships among spirorbin tribes had no support (Figs. 2-1, 2-2, 2-3), so caution was exercised in the presentation of results. However, the lability of clades on the trees was determined by comparing results from different weighting schemes. Trees presented in Figs. 2-1, 2-2 and 2-3 are haphazardly chosen from a suite of most parsimonious topologies that differ in branching patterns among terminal taxa (and not the more basal relationships I focus on here).

Bremer support for both the RCI-weighting and 'taxonomic' weighting were not informative when rescaled (by dividing the extra length values by weighted length/unweighted length of most parsimonious trees) (Gustaffson & Bremer 1994). Differences among weighted lengths for each node were too small relative to the overall values, and therefore support was uniform across all nodes. For this reason, only bootstrap support for trees inferred under these weighting schemes are discussed.

2.3.2 Phylogenetic relationships among the Spirorbinae

The Spirorbinae were monophyletic in all analyses (100% bootstrap support in unweighted and weighted analyses; Figs. 2-1, 2-2, 2-3). Their monophyly was supported by numerous characters: Coiled tubes (26), number of ciliary bands on trochophore larva (40), presence of metatrochophore collar (41), presence and number of anal vesicles (46 and 47), developmental characters (timing of development of collar, branchial, and operculae, 48-50), simultaneous hermaphrodism (51), presence of opercular plates (75), and the presence of an asetigerous region.

The Spirorbinae were nested within Serpulidae in all analyses (Figs. 2-1A, 2-2A, 2-3A). Serpulidae is monophyletic, supported by the presence of a thoracic membrane (20), calcareous tubes (25) and chaetal characteristics (*e.g.*, collar chaetae; 96), among others. The sistergroup to the Spirorbinae was a paraphyletic *Filograna* and *Protula* grade in the unweighted analysis and 'taxonomic' weighted analysis (Figs. 2-2A and 2-3A respectively), and only *Protula* in the RCI weighted tree (Fig. 2-2A; *Filograna* fell within the Serpulinae).

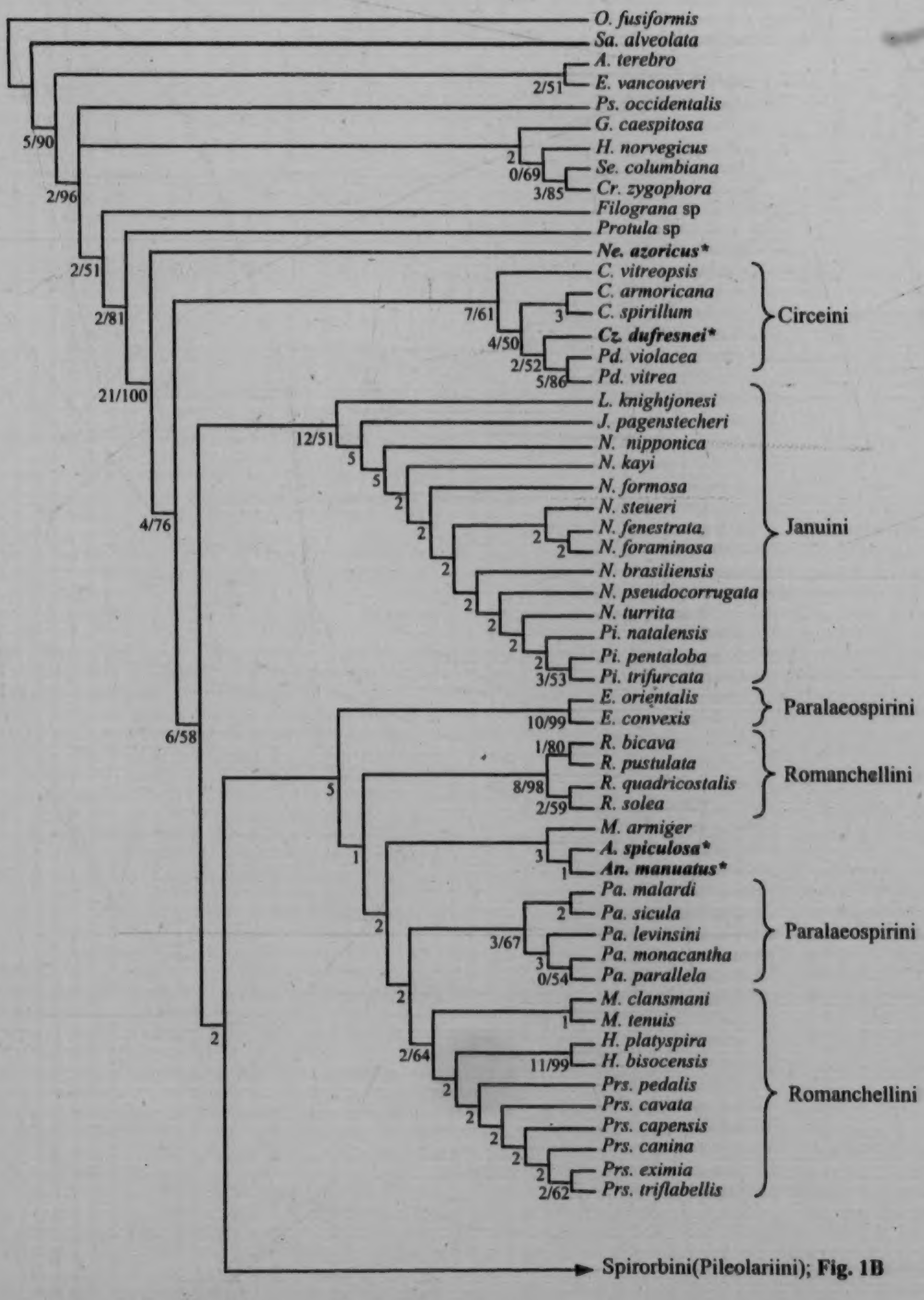
O. fusiformis
Sa. alveolata
A. terebro
E. vancouveri
2/51
Ps. occidentalis
G. caespitosa
H. norvegicus
Se. columbiana
Cr. zygophora
2
0/69
3/85
5/90
2/96
Filograna sp
Protula sp
Ne. azoricus*
2/51
C. vitreopsis
C. armoricana
C. spirillum
Cz. dufresnei*
Pd. violacea
Pd. vitrea
7/61
3
4/50
2/52
5/86
Circeini
2/81
21/100
L. knightjonesi
J. pagenstecheri
N. nipponica
N. kayi
N. formosa
N. steueri
N. fenestrata
N. foraminosa
N. brasiliensis
N. pseudocorrugata
N. turrita
Pi. natalensis
Pi. pentaloba
Pi. trifurcata
12/51
5
5
2
2
2
2
2
2
2
2
2
3/53
Januini
4/76
E. orientalis
E. convexis
10/99
Paralaeospirini
R. bicava
R. pustulata
R. quadricostalis
R. solea
1/80
8/98
2/59
Romanchellini
6/58
5
M. armiger
A. spiculosa*
An. manuatus*
3
1
1
Pa. malardi
Pa. sicula
Pa. levinsini
Pa. monacantha
Pa. parallela
2
3/67
3
0/54
Paralaeospirini
2
2
M. clansmani
M. tenuis
H. platyspira
H. bisocensis
Prs. pedalis
Prs. cavata
Prs. capensis
Prs. canina
Prs. eximia
Prs. triflabellis
1
11/99
2/64
2
2
2
2
2
2/62
Romanchellini
2
Spirorbini(Pileolariini); Fig. 1B

Fig. 2-1A One of 7613 most parsimonious trees (haphazardly chosen) of length 908 based on equal weighting of 148 morphological characters. Continued in Fig. 2-1B. Support values below nodes are decay indices/bootstrap support. Nodes without bootstrap value indicate support of less than 50%. Nodes that collapsed in a strict consensus are indicated by an asterisk.

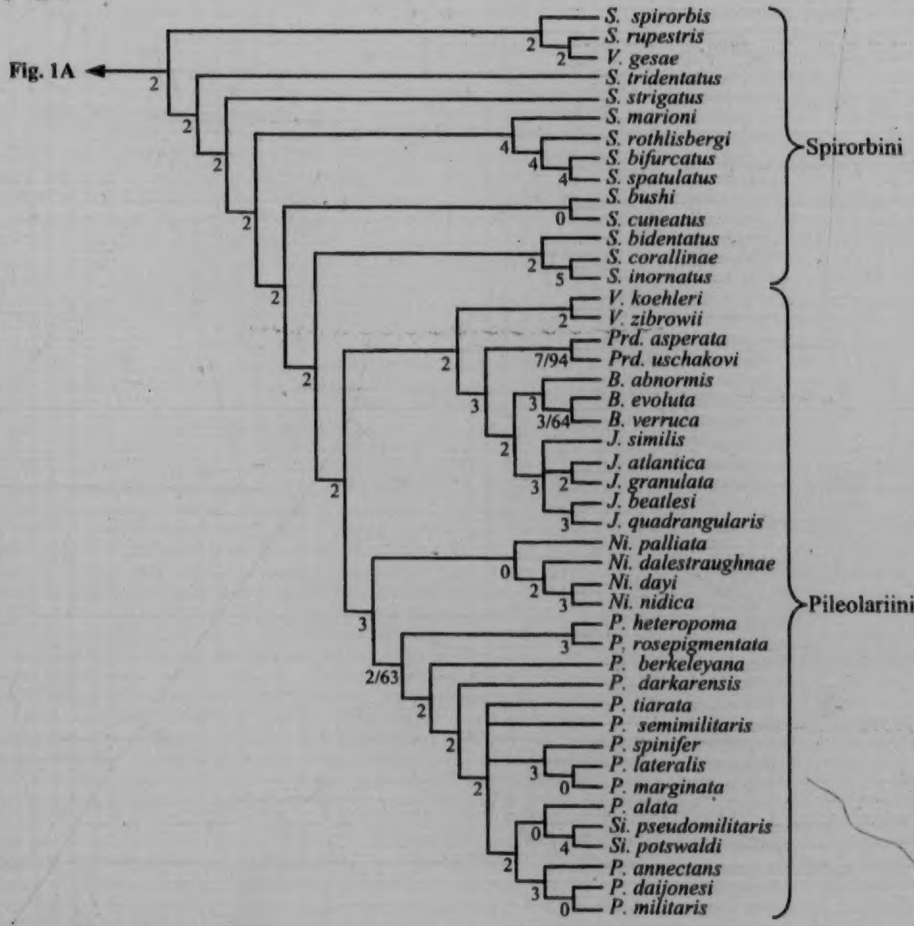
Fig. 1A
S. spirorbis
S. rupestris
V. gesae
S. tridentatus
S. strigatus
S. marioni
S. rothlisbergi
S. bifurcatus
S. spatulatus
S. bushi
S. cuneatus
S. bidentatus
S. corallinae
S. inornatus
V. koehleri
V. zibrowii
Prd. asperata
Prd. uschakovi
B. abnormis
B. evoluta
B. verruca
J. similis
J. atlantica
J. granulata
J. beatlesi
J. quadrangularis
Ni. palliata
Ni. dalestraughnae
Ni. dayi
Ni. nidica
P. heteropoma
P. rosepigmentata
P. berkeleyana
P. darkarensis
P. tiarata
P. semimilitaris
P. spinifer
P. lateralis
P. marginata
P. alata
Si. pseudomilitaris
Si. potswaldi
P. annectans
P. daijonesi
P. militaris
Spirorbini
Pileolariini
7/94
3/64
2/63

Fig. 2-1B Continued from Fig. 2-1A.

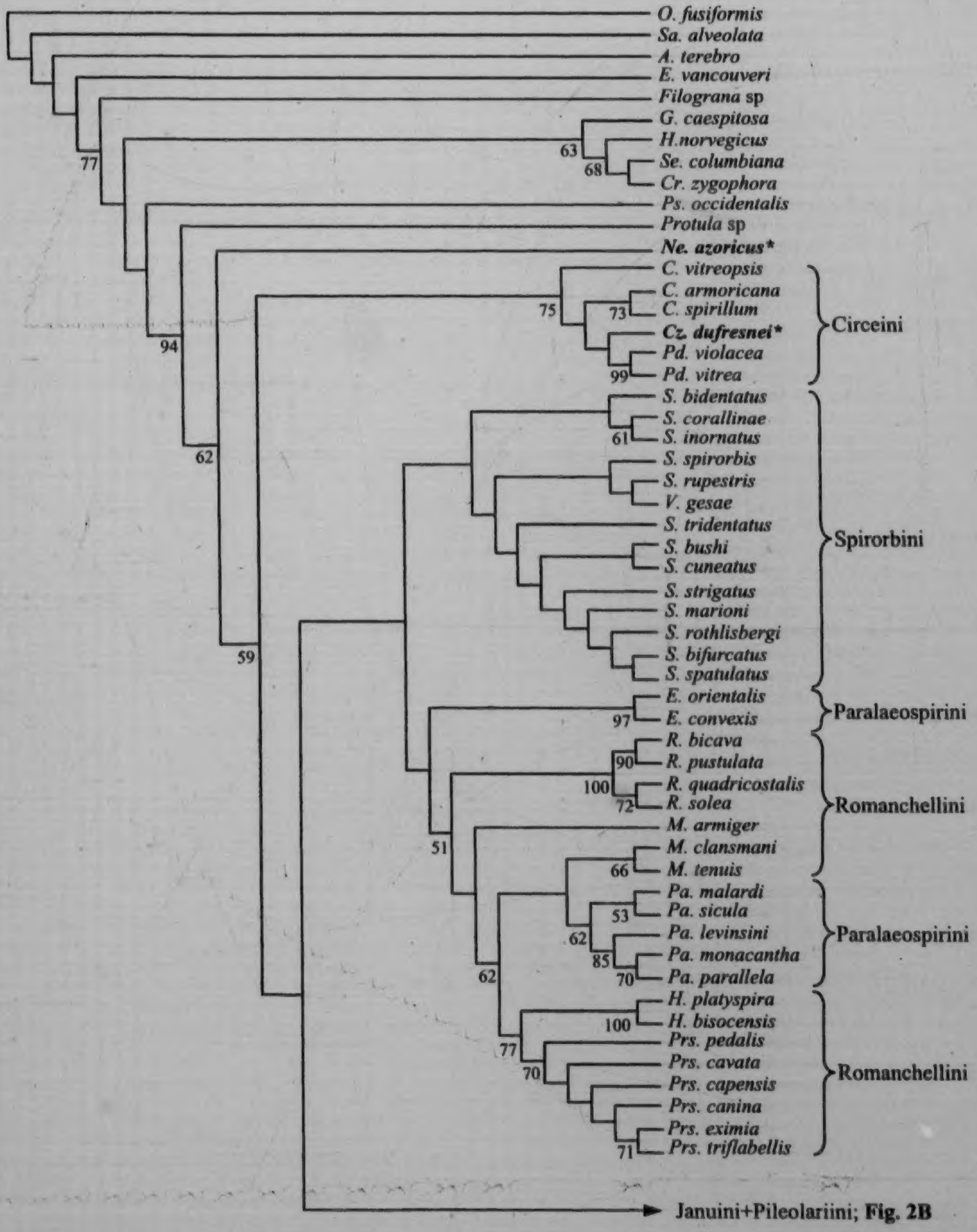
O. fusiformis
Sa. alveolata
A. terebro
E. vancouveri
Filograna sp
G. caespitosa
H.norvegicus
Se. columbiana
Cr. zygophora
Ps. occidentalis
Protula sp
Ne. azoricus*
C. vitreopsis
C. armoricana
C. spirillum
Cz. dufresnei*
Pd. violacea
Pd. vitrea
S. bidentatus
S. corallinae
S. inornatus
S. spirorbis
S. rupestris
V. gesae
S. tridentatus
S. bushi
S. cuneatus
S. strigatus
S. marioni
S. rothlisbergi
S. bifurcatus
S. spatulatus
E. orientalis
E. convexis
R. bicava
R. pustulata
R. quadricostalis
R. solea
M. armiger
M. clansmani
M. tenuis
Pa. malardi
Pa. sicula
Pa. levinsini
Pa. monacantha
Pa. parallela
H. platyspira
H. bisocensis
Prs. pedalis
Prs. cavata
Prs. capensis
Prs. canina
Prs. eximia
Prs. triflabellis
Circeini
Spirorbini
Paralaeospirini
Romanchellini
Paralaeospirini
Romanchellini
77
63
68
94
75
73
99
62
61
59
97
90
100
72
51
66
53
62
85
70
62
100
77
70
71
Januini+Pileolariini; Fig. 2B

Fig. 2-2A One of 3 most parsimonious trees (haphazardly chosen) of length 218.7 based on a scheme in which characters are reweighted by their Rescaled Consistency Indices. Support values below nodes are bootstrap support; Decay indices were found to be uninformative as differences among support values were minimal. Nodes without bootstrap support have support of less than 50%. Continued in Fig. 2-2B.

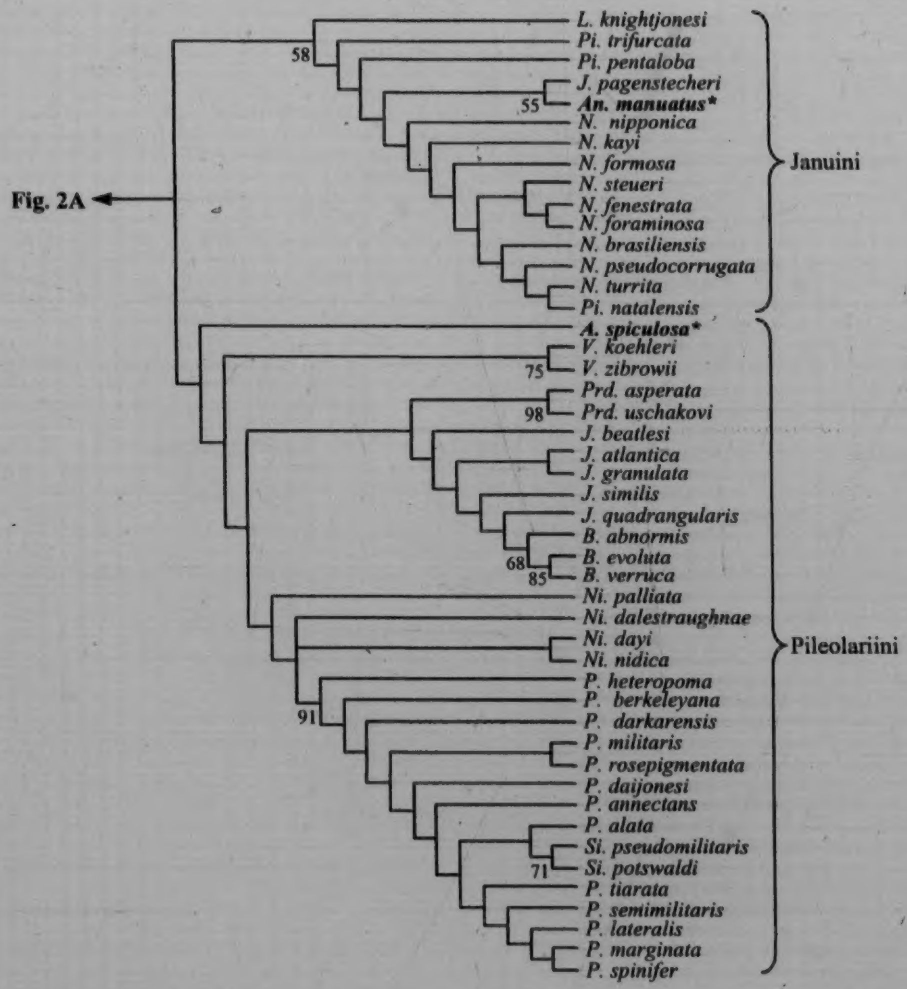
Fig. 2A
58
L. knightjonesi
Pi. trifurcata
Pi. pentaloba
J. pagenstecheri
55
An. manuatus*
N. nipponica
N. kayi
N. formosa
N. steueri
N. fenestrata
N. foraminosa
N. brasiliensis
N. pseudocorrugata
N. turrita
Pi. natalensis
Januini
A. spiculosa*
V. koehleri
75
V. zibrowii
Prd. asperata
98
Prd. uschakovi
J. beatlesi
J. atlantica
J. granulata
J. similis
J. quadrangularis
B. abnormis
68
B. evoluta
85
B. verruca
Ni. palliata
Ni. dalestraughnae
Ni. dayi
Ni. nidica
Pileolariini
P. heteropoma
P. berkeleyana
91
P. darkarensis
P. militaris
P. rosepigmentata
P. daijonesi
P. annectans
P. alata
Si. pseudomilitaris
71
Si. potswaldi
P. tiarata
P. semimilitaris
P. lateralis
P. marginata
P. spinifer

Fig. 2-2B Continued from Fig. 2-2A.

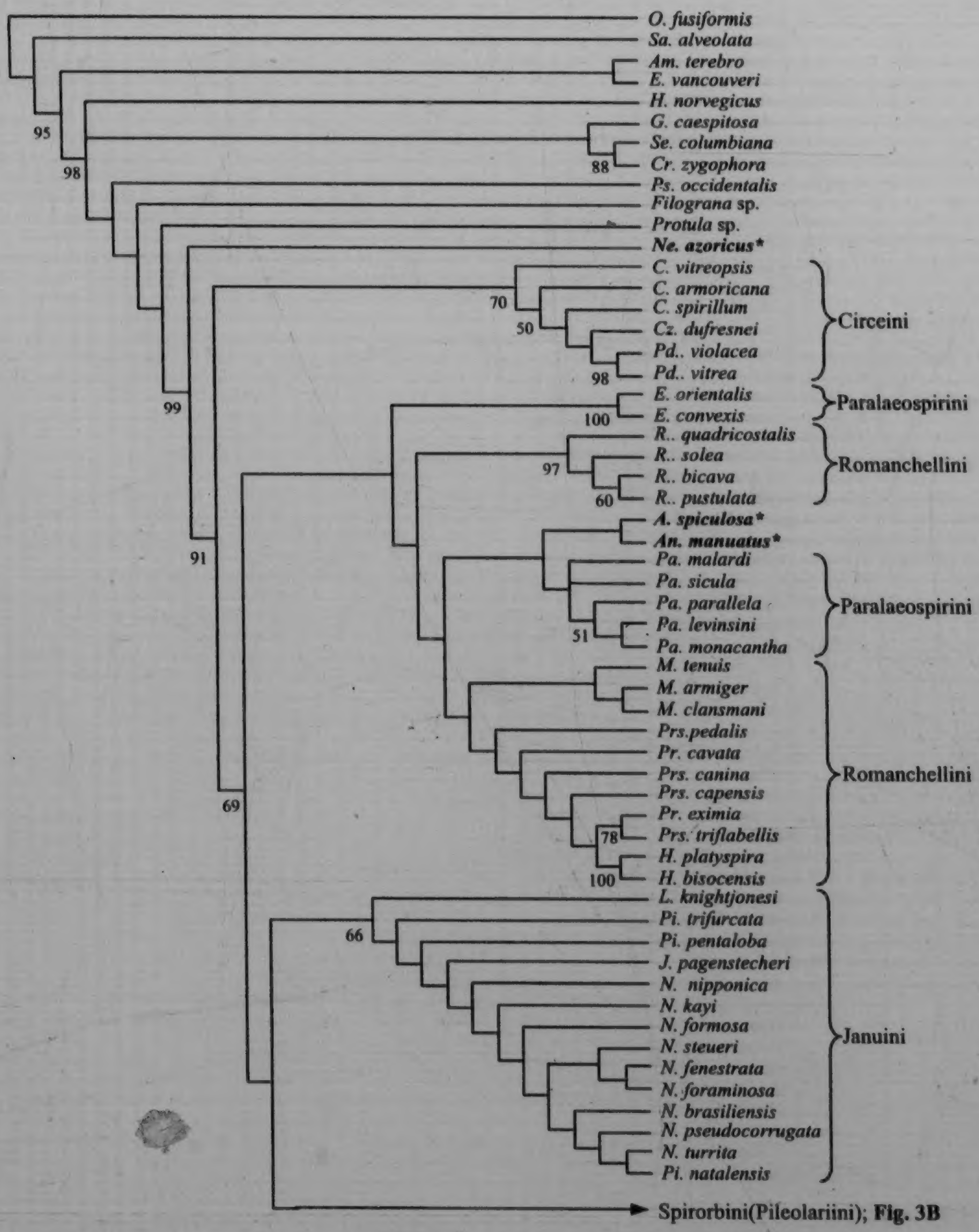
O. fusiformis
Sa. alveolata
Am. terebro
E. vancouveri
H. norvegicus
G. caespitosa
Se. columbiana
Cr. zygophora
Ps. occidentalis
Filograna sp.
Protula sp.
Ne. azoricus*
C. vitreopsis
C. armoricana
C. spirillum
Cz. dufresnei
Pd.. violacea
Pd.. vitrea
Circeini
E. orientalis
E. convexis
Paralaeospirini
R.. quadricostalis
R.. solea
R.. bicava
R.. pustulata
Romanchellini
A. spiculosa*
An. manuatus*
Pa. malardi
Pa. sicula
Pa. parallela
Pa. levinsini
Pa. monacantha
Paralaeospirini
M. tenuis
M. armiger
M. clansmani
Prs.pedalis
Pr. cavata
Prs. canina
Prs. capensis
Pr. eximia
Prs. triflabellis
H. platyspira
H. bisocensis
Romanchellini
L. knightjonesi
Pi. trifurcata
Pi. pentaloba
J. pagenstecheri
N. nipponica
N. kayi
N. formosa
N. steueri
N. fenestrata
N. foraminosa
N. brasiliensis
N. pseudocorrugata
N. turrita
Pi. natalensis
Januini
Spirorbini(Pileolariini); **Fig. 3B**
95
98
88
70
50
98
99
100
97
60
91
51
69
78
100
66

Fig. 2-3A One of 4474 most parsimonious trees (haphazardly chosen) of length 1407 based on the 'taxonomic' weighting (in which traditional taxonomic characters are weighted 5 times more than others). Values below nodes are bootstrap support; nodes without values indicate support of less than 50%. Decay indices were uninformative because differences among support for were too small to be informative. Continued in Fig. 2-3B.

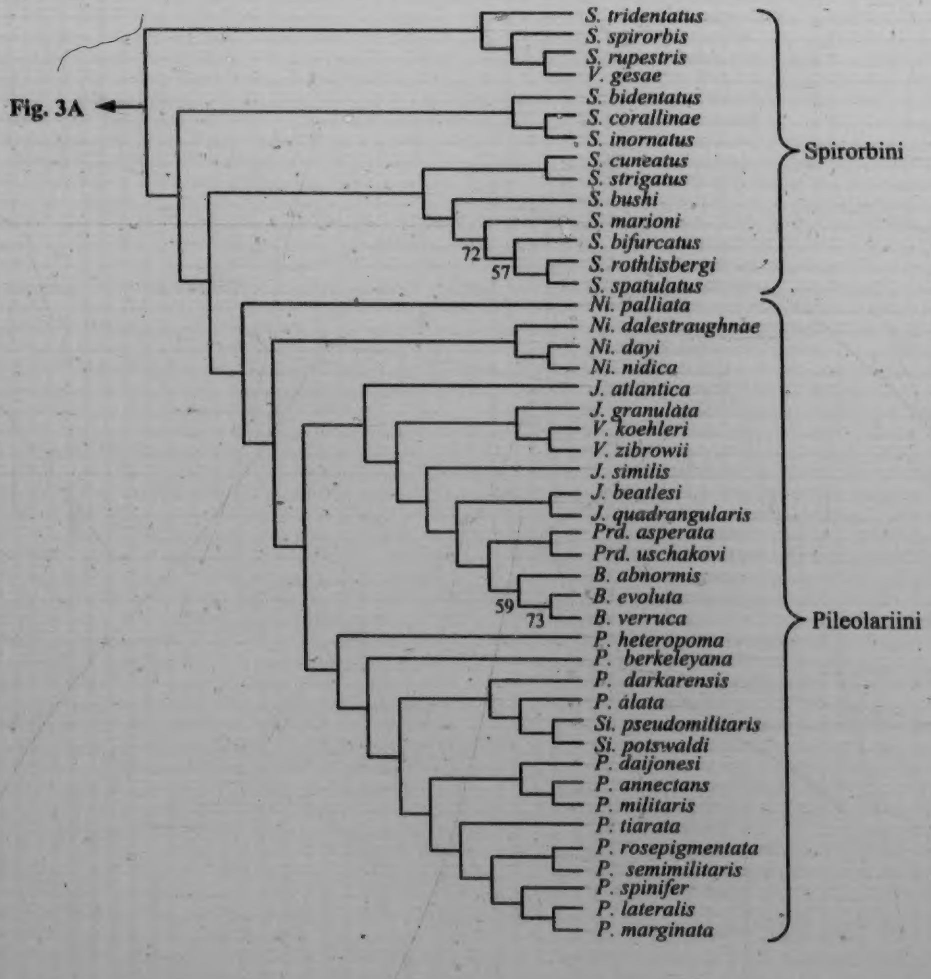
Fig. 3A
S. tridentatus
S. spirorbis
S. rupestris
V. gesae
S. bidentatus
S. corallinae
S. inornatus
S. cuneatus
S. strigatus
S. bushi
S. marioni
S. bifurcatus
S. rothlisbergi
S. spatulatus
72
57
Spirorbini
Ni. palliata
Ni. dalestraughnae
Ni. dayi
Ni. nidica
J. atlantica
J. granulàta
V. koehleri
V. zibrowii
J. similis
J. beatlesi
J. quadrangularis
Prd. asperata
Prd. uschakovi
B. abnormis
B. evoluta
B. verruca
59
73
P. heteropoma
P. berkeleyana
P. darkarensis
P. alata
Si. pseudomilitaris
Si. potswaldi
P. daijonesi
P. annectans
P. militaris
P. tiarata
P. rosepigmentata
P. semimilitaris
P. spinifer
P. lateralis
P. marginata
Pileolariini

Fig. 2-3B Continued from Fig. 2-3A.

Circeini - A monophyletic Circeini (inclusive of *Crozetospira*; incertae sedis taxa discussed later) was basal in all three analyses (>70% bootrap support in all three analyses; decay index of 7 in unweighted analysis). This relationship was based on the arrangement of transverse rows of thoracic and abdominal uncinal teeth (characters 118 and 144 respectively), which are straight in this group and diagonal in all other tribes (where multiple rows exist). Within Circeini, *Circeis* was paraphyletic, with a monophyletic *Paradexiospira* + *Crozetospira* derived within the clade (>70 % bootstrap support in all analyses).

Januini - Januini were also monophyletic (>50% bootstrap support in all analyses, decay index 12 in unweighted analysis) except in the RCI weighted analysis, in which they were rendered paraphyletic by inclusion of *Anomalorbis*. Their monophyly (*Anomalorbis* excluded) was supported by their number of larval ocelli (31), larval attachment glands (45), distinctive brood chambers (57, 59), collar chaetae morphology (96), and pointed thoracic uncinal pegs (120, 122), among others.

The placement of Januini within the Spirorbinae was labile, and depended on the weighting scheme: they were basal in the unweighted analysis (branching off after the Circeini; sister to the remaining spirorbins; Fig. 2-1A), derived in the RCI analysis (members of a derived Januini + Pileolariini clade; Fig. 2-2B) and also derived in the taxonomic weighted analysis (sister to a Pileolariini + Spirorbini clade; Fig. 2-3A).

Leodora was the most basal genus in this tribe in all analyses (Figs. 2-1A, 2-2B and 2-3A), and coils sinistrally (27), whereas all others coil dextrally.

Romanchellini + Paralaeospirini - The Romanchellini and Paralaeospirini were paraphyletic in all analyses, but together formed a monophyletic clade (all analyses; Figs. 2-1A, 2-2A, 2-3A; disregarding inclusion of *Amplicaria* and *Anomalorbis* in the unweighted and taxonomically weighted analysis). They were united by their spermatid arrangement (54), lack of convex abdominal tori (140), and the general asymmetric distribution of uncini and chaetae along their bodies. The placement of this clade in the spirorbin tree was variable based on weighting scheme. In the unweighted analysis, the Paralaeospirinii+Romanchellini were sister to the derived Spirorbini+ Pileolariini clade, branching off after the Januini (Fig. 2-1A). In the RCI-weighted analysis, they formed a basal monophyletic clade with the Spirorbini, branching off directly after the Circeini (Fig. 2-2A). In the taxonomically weighted analysis, they were the most basal group of spirorbins next to the Circeini (Fig. 2-3A).

The relationships within the Paralaeospirini + Romanchellini clade were relatively consistent across weighting schemes. *Eulaeospira* was always the most basal genus, and a *Protolaeospira* + *Helicosiphon* clade the most derived. All genera tended to be monophyletic (including *Romanchella, Protolaeospira, Helicosiphon* and *Paralaeospira*), with the exception of *Metalaeospira*, which was paraphyletic in the unweighted analysis and RCI-weighted analysis (*M. armiger* groups with *Amplicaria* and *Anomalorbis* in the latter analysis).

Spirorbini - Spirorbini also had a labile position within Spirorbinae. The equal weighting and taxonomic weighting schemes both placed them with the Pileolariini in the most derived clade (as a paraphyletic grade, Figs. 2-1B and 2-3B respectively). This placement, although receiving <50% boostrap support in both analyses (decay index 2 in unweighted analysis), was nevertheless supported by the shared presence of single larval attachment glands (42 and 43), the number and distribution of thoracic chaetigers (16-19), clustered spermatids (54), as well as chaetal morphology. The RCI-weighted analysis placed the Spirorbini in a more basal monophyletic clade sister to the Paralaeospirini + Romanchellini (Fig. 2-2A). The genus *Velorbis* (a monotypic genus; *V. gesae*) fell out within *Spirorbis* in all analyses, and had affinity to *S. spirorbis, S. rupestris*, and *S. tridentatus* in all analyses.

Pileolariini - Pileolariini were monophyletic in all analyses (excluding *Amplicaria;* Figs. 2-1B, 2-2B, 2-3B). This relationship was supported by the presence of a paired dorsal thoracic crystalline patch (23 and 24), their epithelial opercular brood chambers (59), and symmetric body characters (*e.g,* symmetric distribution of thoracic uncini, 123). Only in the RCI-weighted analysis was *Amplicaria* included in the Pileolariini, (its traditional placement; Fig. 2-2B).

Pileolariini were derived in all three analyses, and this placement did not depend on weighting scheme. However, its sister group depended heavily on the weighting scheme used: both the unweighted and taxonomically-weighted analyses inferred the sister group to be a paraphyletic Spirorbini; however, the RCI-weighting scheme resulted in the placement of opercular brooders together in the most derived clade, and therefore the sister group of Pileolariini was the Januini.

Incertae sedis *taxa* - *Neomicrorbis azoricus* was basal in all analyses, sister to the remaining spirorbins (unweighted analysis:100% bootstrap support, decay index 21; RCI-weighted analysis and taxonomic-weighted analyses with >95% bootstrap support). This placement was expected because it shares with serpulids a large number of thoracic fascicles and tori (more than any other spirorbin; at least 5;16-19).

Crozetospira dufresnei appeared to have a similarly stable position; it grouped within Circeini in all analyses, sister to the included members of the genus *Paradexiospira* (>50% bootstrap support only in unweighted analysis). This relationship was supported by their collar chaetal morphology (96), distribution of thoracic uncini (124), and lack of abdominal tori on the convex side (139).

Anomalorbis manuatus held a somewhat labile position, grouping with Paralaeospirini + Romanchellini in the unweighted and taxonomic weighting (Figs. 2-1A and 2-3A respectively) and within Januini in the RCI weighting scheme (Fig. 2-2B). In the unweighted and taxonomic weighting schemes, *A. manuatus* grouped with *Amplicaria spiculosa*, a relationship based on the shared presence (with Paralaospirini) of more than four convex thoracic segments (characters 16-19).

2.3.3 Ancestral state reconstructions

The uncertainty in relationships among the major spirorbin clades (their relations being highly dependent on character weighting and weakly supported in all analyses), rendered ancestral state reconstructions impractical and uninformative. Any character optimizations would require subjective assessments of the validity of one weighting scheme over another. However, some conclusions may be drawn from areas of congruence among analyses.

Brooding modes - In general, Circeini were always basal, and therefore the most ancestral spirorbins likely brooded inside their tubes in gelatinous masses. However, the uncertain brooding mode of the basal *Neomicrobis azoricus* makes even this inference suspect. Opercular brooding appears to have arisen more than once, as only the RCI weighted analysis inferred Januini and Pileolariini (both opercular brooders) to be members of the same clade. The Pileolariini more likely evolved from a tube-brooding ancestor belonging to Spirorbini, and the Januini more likely evolved from a Circeini-like ancestor. The brood-stalk of the Romanchellini appears to have arisen at least twice, once in *Romanchella* and again in *Metalaeospira* and *Protolaeospira* and is not a precursor to opercular brooding as hypothesized by Knight-Jones & Thorp (1984).

Evolution of directional asymmetry – These data support Palmer's (1996 a&b, 2004) genetic assimilation hypothesis because the antisymmetric *Neomicrorbis* was basal in all analyses (Figs. 2-1A, 2-2A, 2-3A). Directional asymmetry appears to be the norm in all tribes; however some tribes are more directionally fixed than others. For example, all Paralaeospirini + Romanchellini and Pileolariini (exclusively sinistral) do not have reversals; however other tribes (mostly dextral – Circeini, Spirorbini and Januini) have both sinistral members and records of *situs inversus*.

2.4 DISCUSSION

2.4.1 Clade support and weighting schemes

All trees inferred from these morphological data received little support with the exception of a few genus-level relationships. No basal splits among tribes were supported (Figs. 2-1, 2-2, 2-3); so no strong inferences can be made about relationships among tribes. This uncertainty is illustrated quite clearly by the incongruence among trees inferred under different character-weighting schemes. However, similarities among these analyses may be more revealing than their differences, as these may indicate support for one topology over another. For example, the equal-weighted and taxonomically-weighted analyses yielded more similar trees than the RCI-weighted analysis, and therefore these hypotheses may be more robust.

The inability of morphological data to resolve higher-order relationships is not uncommon among polychaetes (*e.g.*, Rouse & Fauchald 1997, Fitzhugh 1989). The lack of hard parts makes it difficult to score characters, and the phenotypic plasticity of those that do exist (*e.g.*, chaetae, operculae) complicate matters. Thus morphological data may be of limited utility for discerning relationships at the species level in annelids in general, and the Spirorbinae in particular. Further investigation with molecular data is required to test the hypotheses presented here (see Chapter 3, Section 3.4 for a comparison of morphological and molecular phylogenetic hypotheses).

2.4.2 Comparison to the genus-level phylogeny of Macdonald (2003)

This study expanded on a previously published phylogenetic hypothesis, which was constructed at the genus level and included 123 morphological characters. In general, these two studies had a similar lack of resolution among basal branches. Detailed differences between the two studies are discussed in detail in the discussion of relationships among tribes (Section 2.4.4) and within tribes (Section 2.4.5) below. Interestingly, the RCI-weighting scheme yielded similar results in the two studies, namely the single origin of opercular brooding (Pileolariini + Januini; discussed in more detail below), which was not inferred in any other analysis and therefore perhaps should be subject to further examination.

2.4.3 Relationships among tribes

The monophyly of the Spirorbinae was strongly supported (>90% bootstrap in all three analyses). This is not surprising given their morphological distinctiveness among serpuliform polychaetes (*e.g.*, Pillai 1970, Knight-Jones 1981, Ten Hove 1984, Kupriyanova *et al.* 2001). However, their placement within Serpulidae remains questionable, as the analyses unexpectedly revealed more affinity with members of Filograninae rather than Serpulinae, as Ten Hove (1984) hypothesized based on operculum morphology. This relationship is investigated further using molecular data (Chapter 3; Macdonald & Rouse *In review*; Kupriyanova *et al.* 2006; Lehrke *et al.* 2006).

The split of *Neomicrorbis* from the remaining spirorbin genera also occurs in all analyses, and is supported by the presence of a large number of thoracic segments (16-19). The basal position of the Circeini (branching off after *Neomicrorbis*) is also consistent among analyses. Relationships among the remaining tribes were unresolved, but some areas of congruence emerged from the analyses.

All three weighting schemes inferred a derived Pileolariini, and the close affinity of the Paralaeospirini + Romanchellini. Another common trend emerged from the unweighted and taxonomy -weighted analyses: the grouping of Spirorbini with Pileolariini. In the RCI-weighted analysis, the Spirorbini were sister to the Romanchellini + Paralaeospirini. This placement seems unlikely, as it was unique to this weighting scheme, while its grouping with the Pileolariini was supported by the number of thoracic chaetigers (15-17; Knight-Jones & Fordy 1979) and the presence of a single larval attachment gland (Höglund 1951).

Interestingly, the Pileolariini grouped with Januini only in the RCI-weighted analysis. This relationship was also inferred by the genus-level analysis of Macdonald (2003), but has been subsequently reevaluated and rejected by Macdonald & Rouse (*In review*). The findings of Macdonald (2003) were also based on RCI weighting, which is supposed to correct for the use of both binary and multistate characters (Farris 1989) by down-weighting multistate characters (given they will have a greater contribution to overall tree length). However, this weighting scheme may be misleading in this case given the large number of binary characters.

2.4.4 Relationships within tribes

Presuming that the missing characters of *incertae sedis* taxa make their phylogenetic position labile, I will leave discussion of their phylogenetic affinity for later and focus on those genera whose brooding modes (and therefore tribal affinities) are known.

Among the Circeini, the genus *Paradexiospira* is monophyletic, and groups within a paraphyletic *Circeis*. This relationship appears in all three analyses. The genus *Paradexiospira* is distinct from *Circeis* because of its large number of thoracic chaetigers on the concave side (four instead of three; characters 15 and 16). This trend is reversed from the expected reduction in thoracic chaetigers during spirorbin evolution (Knight-Jones & Fordy 1979).

Within Spirorbini, *Velorbis* presents an interesting case; its collar is fused dorsally, while all other Spirorbini (*Spirorbis*) have unfused collars (Knight-Jones & Knight-Jones 1995). The functional significance of this fusion is unknown, but has been suggested by Potswald (1968) to aid in transfer of embryos to the brooding structure. It occurs in two other genera, both tube-brooders and opercular-brooders (*Romanchella* and *Neodexiospira* respectively) and thus appears to have arisen three times independently.

Romanchellini and Paralaeosprinii were, in all analyses, paraphyletic members of the same clade. *Eulaeospira* (traditionally Paralaeospirini, lack a brood-stalk) was the most ancestral genus, and the remaining Paralaeospirini (*Paralaeospira*) were nested within the Romanchellini. Thus the thoracic brood-stalk was either gained independently twice within the clade, or gained and lost again. The close relationship of these two tribes has been noted in the past (*e.g.*, Knight-Jones *et al.* 1971 and Knight-Jones &

Knight-Jones 1994), but it is unclear whether the characters that unite them are convergent due to tube incubation (*e.g.,* loss of abdominal tori on the convex side).

Most members of the Januini belong to the genus *Neodexiospira* (approximately 30 species, all with fused collars), with fewer representatives of *Pillaiospira* (3 species) *Janua* (1 species) and *Leodora* (one species), which have unfused collars (21). *Leodora knightjonesi* is the only sinistral member of the Januini (27), and is the most ancestral member of the clade in all analyses. The phylogenetic position of remaining genera depends on the weighting scheme. Thus despite Januini having the most unique morphology among tribes, within-tribe variation is not well defined (Knight-Jones, Knight-Jones & Kawahara 1975) due to limited morphological variation among genera and species. The taxonomy of this group clearly requires scrutiny.

Among the Pileolariini, however, more morphological variation among genera was evident. In particular, their diverse opercular morphology was explained by Knight-Jones & Thorp (1984) by hypothesizing an increasing trend in the complexity of brood chambers throughout spirorbin evolution, from the 'open nest' of *Vinearia* and *Nidificaria*, to the enclosed chambers of *Pileolaria* and *Simplaria*, to the often highly ornamented and armored chambers of *Protolaeodora, Bushiella* and *Jugaria*. This hypothesis is partly supported by the analysis here. Either *Vinearia* (equal-weighted and RCI-weighted analysis) or *Nidificaria* (taxonomically-weighted analysis) tended to be ancestral. However, *Jugaria, Bushiella*, and *Protolaeodora* also tended to be ancestral to a derived *Pileolaria* + *Simplaria* clade, and thus may more accurately be considered a different lineage of those Pileolariini with enclosed chambers rather than the end point of a gradation in complexity. Genera tended to be monophyletic in all cases, with the exception of *Pileolaria*, which was rendered paraphyletic by *Simplaria*.

The exclusion of *Amplicaria spiculosa* (the sole member of the monotypic genus) from this discussion is not accidental: although traditionally a member of Pileolariini, it may not belong in this taxonomic position. Although *A. spiculosa* was sister to the remaining Pileolariini in the RCI-weighted analysis, it grouped with the Romanchellini + Paralaeospirini in the equal and taxonomic weighted analyses. This is not entirely surprising given their striking differences from other Pileolariini: they have a large number of thoracic chaetigers (rather than just two), release sperm in tetrads instead of clusters, and have an asymmetric distribution of chaetae.

2.4.5 Positions of incertae sedis *taxa*

In the absence of brooding specimens, the tribal affinities of some taxa were unclear. This analysis attempted to discern their phylogenetic positions given the information available, which is limited due to the scarcity of material in all cases. For instance, *Anomalorbis manuatus* Vine, 1972 was described from only a single specimen. It was provisionally placed in the Paralaeospirini due to its large number of thoracic chaetigers (it has five on the convex side and four on the concave side (Vine 1972).

Crozetospira dufresnei Rhzavsky, 1997 was provisionally placed in the Paralaeospirini as well; this was its default position because its asymmetrical distribution of abdominal uncini, and opercular structure indicated it was a tube brooder, and it lacked distinctive characteristics of Romanchellini (brush-like abdominal chaetae) and Spirorbini (two symmetric thoracic tori) and was found much further south than any members of the Circeini. However, the analysis here indicates *C. dufresnei* may in fact be related to the Circeini, based on the arrangement of thoracic uncinal teeth (straight rows instead of diagonal) and coiling direction (dextral), thus it may the most southern member of the Circeini.

Neomicrorbis arzoricus Rovereto 1904 is of great interest because it is the only known member of the Spirorbinae that definitively (as far as we know) exhibits antisymmetry (it has a 50:50 distribution of dextral and sinistral individuals in the field (Zibrowius, 1972; pers. comm.). It was also provisionally placed in Paralaeospirini because of its large number of thoracic chaetigers. However, the analysis here indicates that it occupies a basal position to the remaining spirorbins, and has no direct association to the Paralaeospirini. Its basal placement is supported largely by the presence of up to 7 thoracic chaetigers, which is more than any known extant spirorbin but is common amongst Filograninae.

Significantly, all three of these taxa - *N. azoricus*, *C. dufresnei*, and *A. manuatus* - have a large number of thoracic chaetigers (four to five on concave side). Based on our current understanding of spirorbin evolution, there is a trend to lose thoracic chaetigers; this has been attributed to selection for a long asetigerous region (between the thorax and abdomen, which accommodates tube coiling). A more robust phylogenetic inference is required to infer the true placement of other taxa with a large number of thoracic segments (like the *incertae sedis* discussed above). Some Romanchellini and Paralaeospirini also have a large number of thoracic segments; but these seem to be associated with increasing body size in these groups (the most derived member of their clade being *Protolaeospira*, which is often large-bodied with large brood sizes).

The *incertae sedis* spirorbins are rare; discovery of more specimens, particularly brooding ones, would provide valuable clues in discerning spirorbin ancestral states. There are intriguing possibilities: perhaps these species do have affiliations with existing tribes, and their large number of thoracic chaetigers is a character reversal. Alternatively, perhaps they do not belong to any existing tribe but possess as yet undescribed brooding modes. Discovery of such traits may help us place evolutionary transitions among existing tribes more accurately. The latter seems a possibility, as their morphology does not place them comfortably in any group (*e.g.*, Zibrowius 1972, Rhzavsky 1997, Vine 1972, Knight-Jones & Fordy 1979).

2.4.6 Evolution of body size

It is assumed that body size has changed unidirectionally in the Spirorbinae (*e.g*, Pillai 1970). Members of most tribes have relatively uniform body sizes (2-3 mm in spiral diameter). However, there is evidence for an evolutionary increase in body size in most clades. For instance, many Romanchellini have large body sizes (more than 4 mm in spiral diameter) (*e.g., Metalaeospira tenuis* (Knight-Jones 1973), many *Protolaeospira* spp. (*e.g.*, Knight-Jones *et al.* 1979) and *Helicosiphon (*Knight-Jones *et al.* 1971). The analysis here indicated that these may be derived from ancestors with smaller body sizes (namely *Eulaeospira* spp. and *Paralaeospira* spp.). The same observation is true for the Pileolariini, which includes large members (*e.g., Simplaris potswaldi* and *Bushiella* spp.). Thus extant members of the Spirorbinae are not necessarily miniaturized compared to their ancestors.

2.4.7 Evolution of directional asymmetry

The basal placement of the antisymmetric *Neomicrorbis azoricus* as the sister-group to all remaining spirorbins (in all three analyses; Figs. 2-1, 2-2, 2-3) lends support to the hypothesis that the directional coiling asymmetry of most extant members of the Spirorbinae evolved through genetic assimilation (phenotype-precedes-genotype) as described by Waddington (1953). In other words, because in virtually all heritability studies with antisymmetric species direction of bias was not inherited (Palmer 2004), the evolutionary transition from antisymmetry (direction not inherited) to directional asymmetry (direction inherited) in spirorbin tubeworms may represent another example of genetic assimilation (phenotype-precedes-genotype; Palmer 2004). However, in the absence of other antisymmetric species, we cannot say with certainty that antisymmetry is a basal condition in the Spirorbinae, or arose independently in *N. azoricus*. The search for more antisymmetric spirorbins may be difficult, as antisymmetry may often be an evolutionarily short-lived character-state (Palmer 2004), possibly due to selection for more canalized and stable development.

The vast majority of the Spirorbinae, therefore, have fixed coiling directions (or at least a low rate of *situs inversus*). Some clades seem phylogenetically constrained to a particular coiling direction; this is especially true of the sinistral Pileolariini and Paralaeospirini + Romanchellini. The Januini, Circeini, and Spirorbini have mostly dextral species but also some sinistral members as well. The fact that no tribe is exclusively dextral may indicate that sinistrality is more likely to become fixed, or perhaps dextrality is more prone to reversals. Developmental and ecological studies in differences among sinistral and dextral forms may reveal any selective differences between coiling morphs (*e.g.*, see Chapter 5, Section 5.4).

Curiously, although asymmetry is inherent in all spirorbin body plans, there appears to be an evolutionary trend of increasing *symmetry* of the spirorbin body, most notably in the thoracic chaetigers. The Pileolariini and Spirorbini, members of the most derived clade of Spirorbinae, have remarkably *symmetrical* bodies with respect to the number of thoracic chaetigers and chaetal characteristics (the number and sizes of thoracic and abdominal tori are similar on both sides of the worm). However, tube brooders, such as the Romanchellini, Paralaeospirinii and Circeini, may simply have an asymmetrical distribution of uncini and chaetae to accommodate broods inside their tubes. This seems likely given that the opercular-brooding Januini appear to have symmetrical bodies independent of the Pileolariini. However, this does not explain why the derived tube-brooding Spirorbini have remarkably *symmetrical* chaetation, or why that of the more basal opercular-brooding *Amplicaria spiculosa* is remarkably *asymmetrical*. Perhaps these represent instances of developmental constraint.

2.4.8 Evolution of brooding modes

It remains unclear how the basal species of Spirorbinae brooded their embryos (or if it did at all) given that *Neomicrorbis azoricus*, the sister-group to the remaining Spirorbinae, has an unknown mode of brooding (*incertae sedis*). However, the proto-spirorbin was likely a tube-brooder, given that brooding Filograninae (sister-group to extant spirorbins) incubate embryos inside their tubes (Kupriyanova *et al.* 2001), and that opercular brooders occupy more derived positions on inferred trees (Figs. 2-1, 2-2, 2-3).

Contrary to earlier hypotheses (Macdonald 2003; Knight-Jones & Thorp 1984), opercular brooding appears to have multiple origins. In only one analysis (RCI weighting; Fig. 2-2) did the opercular-brooding Januini and Pileolariini group together in a single clade. In the other two analyses, Januini and Pileolariini are independent lineages; thus opercular brooding may have evolved more than once (Figs. 2-1 and 2-3). Consideration of *Amplicaria spiculosa* further complicates the story: it is not placed within Pileolariini (its traditional classification), but within Romanchellini + Paralaeospirini (equal and taxonomic weighting). Thus opercular brooding could have evolved independently three times. Careful comparison of larval and opercular morphology of *A. spiculosa* may help us better understand its relationship to other opercular brooders.

The potential for multiple origins of opercular brooding supports the hypothesis that it may be advantageous; however, tube brooding may also have multiple origins. This study indicates it has evolved independently at least 3 times - in Circeini, Spirorbini, and Romanchellini + Paralaeospirini. The lack of resolution among tribes makes the evolutionary sequence impossible to discern. Nevertheless, there are some indications that our hypotheses of spirorbin evolution need to be challenged.

The method of tube incubation in the Circeini (embryo mass adhering to the tube wall) was ancestral in all analyses. This finding is contrary to the assumption that Paralaeospirini are the most ancestral members of Spirorbinae, which was based on their large number of thoracic segments (Rhzavsky 1997, Knight-Jones & Fordy 1979, Vine 1975), a character they share with the remaining Serpulidae. The Paraplaeospirni are instead paraphyletic members of a clade containing the Romanchellini. Thus their brooding mode, in which embryos are incubated inside the tube in loose strings (not attached to adult structures), was just as likely to result from the evolutionary loss of the thoracic brood-stalk as an ancesntral form. *Metalaeospira* (traditionally Romanchellini) appears to be a transitory form between the Paralaespirini and Romanchellini because they have a reduced brood-stalk [termed an 'oviducal funnel' by Knight-Jones & Knight-Jones (1994).

The placement of Romanchellini+Paralaeospirini as sister to the Pileolariini(Spirorbini) clade in two of three analyses (RCI excluded) is consistent with the hypothesis of Knight-Jones & Thorp (1984) that the thoracic brood-stalk is an evolutionary precursor to an opercular brood chamber. This inference rests on the discovery that the brood-stalk is hollow (Knight-Jones *et al.* 1972), and the assumption that it is a conduit for transmission of fertilized eggs. This mode of transmission is suggested as a possible route for the entrances of fertilized eggs into opercular brood chambers of both the Pileolariini and Januini (*e.g.*, Potswald 1968). However, it is just as likely, judging from the data presented here, that the brood-stalk is a reduction of a Januini-type opercular stalk (based on the equal-weighted analysis) - but Pillai (1960) notes the thoracic brood-stalk is in a different position from the rest of the radioles (closer to the midthorax rather than part of the opercular crown) and concludes these structures are unrelated. Nonetheless, the Pileolariini chamber appears not to be homologous with either the brood chamber of the Januini nor the thoracic brood-stalk of the Romanchellini, given their derived position on the tree.

Hypotheses regarding the evolution of both tube- and opercular-brooding should thus be reevaluated. Macdonald (2003) stated: "opercular brooding resulted in a diversification event by overcoming constraints on reproduction imposed by in-tube incubation. The potential disadvantages to opercular brooding – such as the susceptibility of larvae to predators or reduced number of young – may not be as severe as tube-incubation constraints". However, tube-brooding may have its own advantages, given that it has arisen independently from opercular-brooding ancestors as well. Study of the developmental correlates of miniaturization and the costs and benefits of tube versus opercular incubation may shed some light on these evolutionary transitions (Chapter 4). Further resolution of the evolution brooding modes using molecular phylogenetics may help us answer some questions evoked in this study (Chapter 3) and therefore help us better understand the evolution of these fascinating, if diminutive, tubeworms.

2.5 CONCLUSIONS

Spirorbinae are characterized by their diverse modes of brooding. The evolutionary transitions among them have been the subject of much speculation. This study attempts to reconstruct the evolutionary history of the subfamily at the species level in order to generate a robust hypothesis of their evolution, and place this speculation in a phylogenetic framework. I find that opercular brooding appears to have arisen multiple times within the group (at least twice, but possibly three times), and that tube brooding also has multiple origins. However, the deep relationships among tribes remain unresolved, thus the need for further investigation using molecular data is apparent. This study is the first step towards understanding the detailed evolutionary history of these intriguing tubeworms.

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CHAPTER 3.

MOLECULAR PHYLOGENY OF THE SPIRORINAE BASED ON 28S & 18S rDNA AND TOTAL EVIDENCE: IMPLICATIONS REGARDING THE EVOLUTION OF BROODING MODES *

3.1 INTRODUCTION

The diversity of reproductive modes in the calcareous-tube-dwelling serpulid polychaetes (traditionally Serpulinae Rafinesque 1815, Filograninae Rioja 1923 and Spirorbinae Chamberlin 1919) is intriguing; Spirorbinae in particular show a variety of novel brooding structures (Bailey 1969; Kupriyanova *et al.* 2001). Though they have distinctive spirally-coiled tubes and conisistent small size (2-5 mm body length), they are a diverse (approximately 130 described species), clearly monophyletic group (Pillai 1970; Macdonald 2003). As such they present an opportunity to test assumptions about the evolution of reproductive traits. The morphological distinctiveness of Spirorbinae among serpuliform polychaetes prompted Pillai (1970) to elevate them from a subfamily within Serpulidae to family rank, Spirorbidae. This spurred development of taxonomic structure within Spirorbidae into six subfamilies (see Knight-Jones 1978; Knight-Jones & Fordy 1979). These subfamilies were defined by brooding modes elucidated by Bailey

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(1969). However, recognition of Spirorbidae renders Serpulidae paraphyletic (Fitzhugh 1989) and therefore I retain the name Spirorbinae and refer to the subfamilies erected by Knight-Jones (1978) as tribes (note the correct form of these names - Spirorbiinae and Spirorbiidae - is not used here for stability purposes. For this reason I also use the tribe name Spirorbini instead of Spirorbiini).

Four of these tribes brood inside their tubes: Paralaeospirini Knight-Jones, 1978 (*Paralaeospira*, *Metalaeospira* and *Eulaeospira*) brood a single embryo string loose within the tube; Circeini Knight-Jones, 1978 (*Circeis* and *Paradexiospira*) embryo strings adhere to the tubes walls in a gelatinous matrix; in Spirorbini Chamberlin, 1919 (*Spirorbis*), the embryo string is attached to the tube posteriorly by an epithelial string; and Romanchellini Knight-Jones, 1978 (*Helicosiphon*, *Romanchella*, and *Protolaeospira*) has a thoracic attachment stalk connecting the brood mass to the adult body. This stalk has been suggested to be homologous with a recessed radiole (Knight-Jones and Thorp, 1984). The remaining two tribes; Januini Knight-Jones, 1978 (*Janua*, *Pillaiospira*, *Leodora*, *Neodexiospira*) and Pileolariini Knight-Jones, 1978 *Amplicaria*, *Nidificaria*, *Vinearia*, *Pileolaria*, *Bushiella*, *Jugaria*, *Simplaria*), use their plug-like radiole, or operculum, as a brood chamber; extending their brood out of the tube mouth. The two opercular-brooding tribes are morphologically distinct (Knight-Jones & Thorp 1984). The brood chamber of Januini is cylindrical and cuticular. It is formed by the swelling and subsequent degeneration of the opercular ampulla and can be used for only one brood (embryos released by dehiscence of the brood chamber). The Pileolariini brood chamber is formed by invagination of the ampulla, resulting in walls constructed of a double epithelium and a pore for embryo release (and possibly entrance; Potswald 1968, 1977).

These can be used for more than one brood. Opercular brooders comprise more than 70% of described Spirorbinae species (Knight-Jones *et al.* 1979; 1/5 of which belong to the Januini; Kupriyanova *et al.* 2001), which has prompted speculation of the possible advantages of opercular brooding (*e.g.*, Hess 1993; Macdonald 2003).

A morphological phylogenetic study gave little resolution to the relationships among spirorbin tribes (Macdonald 2003; Section 2.3.1). Some evidence suggests opercular brooding arose at least twice, but this conclusion is highly dependent on the weighting scheme used. Macdonald's (2003) genus-level analysis suggests opercular brooders form a single clade, with Januini as a derived clade within Pileolariini. A Romanchellini+Paralaeospirini clade was hypothesized to be the sister-group to opercular brooders; which lended some support to the idea that the romanchellin thoracic brood stalk is a 'step' in evolution towards opercular brooding (suggested by Knight-Jones & Thorp 1984). The tube-brooding tribes were formed a basal grade or to be sister group to the remaining Spirorbinae (Macdonald 2003).

The purpose of this study is to assess, using molecular data in addition to existing morphological data, the phylogenetic hypotheses generated by Macdonald (2003, Figs. 2-1,2 & 3). Phylogenetic analyses were conducted with 18S and 28S rDNA, genes that have proven useful for broader systematic studies of polychaetes [*e.g.*, Autolytinae (Nygren & Sundberg, 2003); Siboglinidae (Rousset *et al.* 2004); Orbiniidae (Bleidorn, 2005) and Arenicolidae (Bleidorn *et al.* 2005)]. These data should improve the resolution of the existing phylogeny of Spirorbinae and provide a framework to interpret and reassess our ideas of the evolution of their morphology. It may also help us answer the following questions: (1) Did opercular brooding evolve more than once? and (2) What is the ancestral brooding mode of Spirorbinae?

This work is in review in *Molecular Phylogenetics and Evolution,* authored by myself and Greg Rouse (South Australia Museum, Adelaide, South Australia). He aided in collection of various spirorbin specimens and contributed some outgroup sequences and three 28S spirorbin sequences.

3.2 METHODS AND MATERIALS

3.2.1 Taxon sampling

The18s rDNA data set consisted of 43 taxa; 30 ingroup spirorbin species and 13 outgroup taxa. 34 were newly sequenced for this study; 8 sequences were mined from Genbank and one (*Pomatoceros triqueter*) was provided by colleagues (J. Lehrke and C. Bleidorn, FU Berlin). A subset of these taxa comprised the 28s data set (41 taxa; 35 new sequences) (Table 3-1).

Outgroup taxa (13 in 18s data set; 11 in 28s) included representatives of Oweniidae, Sabellariidae, Sabellidae and Serpulidae (Serpulinae and Filograninae). Ingroup terminals encompassed the diversity of brooding modes observed in the Spirorbinae to date (Knight-Jones & Fordy 1979). The missing genera were either rare, with unknown brooding modes (*Neomicrorbis, Crozetospira, Anomalorbis*), or had clear morphological affinity with other genera represented here (*Nidificaria, Protolaeodora, Helicosiphon, Pillaiospira,* and *Leodora*). These genera were not included in this study owing to difficulties obtaining rare material in appropriate preservative.

3.2.2 Collection and preservation

Specimens were predominantly collected in British Columbia, Canada, Scandinavia including Iceland and Norway, and southeastern Australia (Table 3-1). Collections were done between 2001 and 2004, when specimens were preserved in 95% ethanol for DNA extraction, and formalin for identification, morphological study and voucher material. Samples remained frozen at -80°C until preparation for sequencing.

3.2.3 DNA extraction, amplification and sequencing

Worms were removed from their tubes and sliced longitudinally. Visible traces of the digestive tract of the non-operculum bearing half of the worm were removed, and the remaining tissue was used in subsequent DNA extraction. The remaining half, including the diagnostic operculum, was saved as a voucher specimen and deposited at the South Australia Museum (SAM), Adelaide, South Australia (Table 3-1).

To remove traces of ethanol, the tissue was rinsed in 1X Phosphate Buffered Saline (PBS) three times, and left to soak for approximately 1 hour at the last rinsing step. Genomic DNA was extracted using a Qiagen DNA Mini Kit (tm) (Qiagen Inc.) and eluted in 50-100 µL of sterile distilled water.

Two nuclear genes were amplified: 18s and 28 ribosomal DNA. 18s rDNA was amplified in 2 overlapping fragments of approximately 1100 bp each using the primers 18s1F & 1R and 18s2F & 2R (Nygren & Sundberg, 2003; Medlin *et al.* 1988). In some cases, reamplification using nested PCR (see Table 3-2 for list of internal primers) was necessary. 28s rDNA was amplified in either a 1000 bp fragment (D1 plus subsequent region with primers 28sF (Boore & Brown 2000) and Po28R4 (Struck *et al.* 2005); the

Table 3-1 List of species analyzed. Asterisks indicate specimens collected by G. Rouse. Voucher numbers are references for South Australia Museum (SAM) collections.

Taxon	Source	Voucher Number	Accession Number	
			18s	28s
Oweniidae				
Owenia fusiformis	GenBank	-	AF448160	AF185152
Sabellariidae				
Sabellaria alveolata	GenBank	-	AY340442	AY340416
Idanthyrsus pennatus	GenBank	-	–	AF185174
Sabellidae				
Amphiglena terebro	GenBank	-	–	AF185150
Eudistlyia vancouveri	Victoria Harbor, BC, Can.	E3502	–	D242547
Sabella spallazani	GenBank	-	AY436350	–
Sabella pavonina	GenBank	-	–	AY340420
Serpulinae				
Crucigera zygophora	Barkley Sound, BC, Can.	E3503	DQ242543	DQ242577
Ficopomatus enigmaticus	GenBank	-	AY577889	–
Galeolaria caespitosa	GenBank	-	AB106257	AF185151
Hydroides norvegica	GenBank	-	AY611452	AY611439
Pomatoceros lamarcki	GenBank	-		
Pseudochitinopoma occidentalis	Barkley Sound, BC, Can..	E3501	DQ242542	DQ242575
Serpula columbiana	Barkley Sound, Can.	E3505	–	DQ242576
Serpula vermicularis	GenBank	-	AY395721	–
Filograninae				
Salmacina sp.	Edithburgh SA Aus.	E3499	DQ242544	DQ242578
Protula sp. VR-2004	GenBank	-	AY611453	AY611440
Spirorbinae				
Pileolariini				
Amplicaria spiculosa	Whyalla, SA, Aus.	E3490	DQ242560	DQ242579
Bushiella abnormis	Barkley Sound, BC, Can.	E3488	DQ242563	DQ242598
Jugaria quadrangularis	Barkley Sound, BC, Can.	E3479	DQ242564	DQ242599
Pileolaria marginata	Barkley Sound, BC, Can.	E3478	DQ242565	DQ242594
Pileolaria militaris	Pt. Cartwright, QLD, Aus.	E3492	DQ242567	DQ242593
Pileolaria sp. 1 (orange eggs)	Whyalla, SA, Aus.*	E3493	DQ242562	DQ242596
Pileolaria sp. 3 (gold eggs)	Rapid Bay, SA, Aus.	E3494	DQ242568	DQ242597
Simplaria potswaldi	Barkley Sound, Can.	E3504	DQ242566	DQ242595
Vinearia koehleri	Pt. Cartwright, QLD, Aus.	E3475	DQ242561	DQ242592
Januini				
Janua pagenstecheri	Sangerdi, Iceland*	E3506	DQ242548	DQ242585
Neodexiospira brasiliensis	Barkley Sound, BC, Can.	E3498	DQ242550	DQ242586
Neodexiospira nipponica	Barkley Sound, BC, Can.	E3486	DQ242549	DQ242587
Neodexiospira steueri	Encounter Bay, SA, AUS*	E3523	DQ242551	DQ242588

Table 3-1 cont'd.

Taxon	Source	Voucher Number	Accession Number	
			18s	28s
Paralaeospirini				
Paralaeospira sp.	Encounter Bay, SA, Aus.*	E3485	DQ242555	DQ242580
Eulaeospira convexis	North Bondi, NSW, Aus.*	E3496	DQ242552	DQ242582
Eulaeospira 'orientalis'	Encounter Bay, SA, Aus.*	E3495	DQ242553	DQ242581
Romanchellini				
Protolaeospira eximia	Barkley Sound, BC, Can.	E3482	DQ242556	DQ242584
Protololaeospira tricostalis	Bondi, NSW, Aus.*	E3487	DQ242557	DQ242606
Protolaeospira capensis	Bondi, NSW, Aus.*	E3484	DQ242558	DQ242607
Romanchella quadricostalis	Kangaroo Island, SA, Aus.*	E3491	DQ242559	DQ242608
Metalaeospira tenuis	Port Lincolin, SA, Aus.*	E3480	DQ242554	DQ242583
Circeini				
Circeis armoricana	Barkley Sound, BC, Can.	E3476	DQ242545	DQ242589
Circeis spirillum	Stykkishlómør, Iceland*	E3507	DQ242546	DQ242590
Paradexiospira vitrea	Barkley Sound, Can.	E3483	DQ242547	DQ242591
Spirorbini				
Spirorbis bifurcatus	Barkley Sound, BC, Can.	E3489	DQ242569	DQ242600
Spirorbis bushi	PJs Pets, Edm., AB, Can.	E3481	DQ242570	DQ242605
Spirorbis corallinae	Finnøy, Norway *	E3497	DQ242572	DQ242603
Spirorbis rupestris	Finnøy, Norway *	E3500	DQ242571	DQ242601
Spirorbis spirorbis	Sangerdi, Iceland*	E3357	AY57788	DQ242604
Spirorbis tridentatus	Sjøhus Finnøy, Norway*	E3477	DQ242573	DQ242602

majority of taxa), or when this amplification failed, a 400 bp region (D1 only with primers 28sF and 28sR: *Protolaeospira tricostalis*, *Protolaeospira capensis* and *Romanchella quadricostalis*) (see Table 3-2 for primer sequences).

PCR reactions were 25 µL and contained the following: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2-4 mM MgCl2, 0.5 (M each primer, 100 (M each dNTP, 1.5 Units Taq (M. Pickard, University of Alberta) and 20-100 ng template DNA (usually 1-2 µL). For taxa that did not amplify well, 2% DMSO was added to the reaction mix, which often resulted in a successful amplification. The following PCR temperature profiles were used: 18s - 95°C for 3 min, 40 cycles of 94°C for 30 sec, *a*°C for 1 min, 72°C for 1.5 min, and a final extension step at 72°C for 10 min; where a = 47-49°C. For 28s, the cycling protocol was the same except the first extension step was reduced to 1 min at 72°C, and a = 46-48°C.

Amplification products were separated via electrophoresis on a 1.1% agarose gels in TAE buffer, stained with ethidium bromide. PCR products were either purified directly with a PCR Purification Kit (Qiagen Inc.) or bands were excised from the gel and purified with a QIAQuick™ Gel Extraction Kit (Qiagen Inc.). Elution was done in sterile distilled water in both cases.

Sequences were obtained directly with the BigDye v 3.1 Cycle Sequencing Kit (Applied Biosystems). Full reactions were 20 µL: 2µL Big Dye, 6 µL buffer (200 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$), µL 1µM primer and 1-6 µL PCR product. Cycle sequencing was done according to the manufacturer's instructions, and separated on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Basecaller v.3.4.1 was used to read the chromatograms and GeneTool 2.0 to assemble gene fragments.

Table 3-2 Primers used in this study for amplification and sequencing of 18s and 28s rDNA. Abbreviations are Forward and Reverse (F/R) and PCR or Sequencing (P/S).

Primer	Position	F/R	P/S	Primer Sequence (5'-3')	Reference
18s1F	1-20	F	P, S	AYCTGGTTGATCCTGCCAGT	Medlin *et al.* 1988
18s2F	673-688	F	P, S	GTTGCTGCAGTTAAA	Nygren *et al.* 2003
18s1R	1214-1235	R	P, S	TASGACGGTATCTGATCGTCTT	Nygren *et al.* 2003
18s2R	2050-2073	R	P, S	ACCTTGTTACGACTTTTACTTCCTC	Nygren *et al.* 2003
18sFA	80-104	F	P, S	GGCTCATTAAATCATAYGTGATTT	This study
18sFB	333-353	F	S	GCGACRTATCTTTYAAGCGTA	This study
18sFC	1085-1100	F	S	AGGGACTGCCGGGGGC	This study
18sFD	1414-1434	F	S	TTAATTTGACTCAACACGGG	This study
18sFE	1716-1738	F	S	AGGTCTGTGATGCCCTTAGATGT	This study
18sRA	151-174	R	S	GCTCTAGAATTRCCACAGTTATCC	This study
18sRB	473-493	R	S	TTTRCGCGCCTGCGGCCTTCC	This study
18sRC	969-983	R	S	TTCYATTATTCCATG	This study
18sRD	1551-1569	R	S	AGAGTCTCGTTCGTTATCG	This study
18sRE	1933-1960	R	S	CCATCCAATCGGTAGTAGCGACGGG CGG	This study
18sFCR	1085-1100	R	P, S	GCCCCCGGCAGTCCCT	This study
28sF	8-26	F	P, S	ACCCSCTGAAYTTAAGCAT	Brown *et al.* 1999
28s R		R	P, S	AACTCTCTCMTTCARAGTTC	Brown *et al.* 1999
Po28R4		R	P, S	GTT CAC CAT CTT TCG GGT CCC AAC	Struck *et al.* 2005
Po28F		F	S	GTT GGG ACC CGA AAG ATG GTG AAC	

3.2.4 Alignment

Sequences were aligned using Clustal W (Thompson *et al.* 1994) with gap opening and extension penalties of 15 and 8 respectively (pairwise and multiple). These values minimized the number of ambiguously aligned bases. Alignments were edited by eye in MacClade 4.07 (Maddison & Maddison 2005). Nucleotides positions that could not be aligned unambiguously were excluded. Manipulation of gap opening and extension penalties affected only ambiguous regions and did not change the inferred topology. The original alignments were 2184 bp (18s) and 1238 bp (28s). With ambiguous sites excluded, the alignments were 1585 bp (18s) and 952 bp (28s). It is not unusual among polychaetes to have ~20% of bases unalignable in non-protein coding genes such as 18s and 28s rDNA (*e.g.*, Bleidorn *et al.* 2003, Brown *et al.* 1999 respectively). The combined 18s +28s data set consisted of 2537 bp. Alignments are available from TreeBase (www.treebase.org).

3.2.5 Morphological data

The morphological matrix used in this study is based on that of Macdonald (2003; a genus-level analysis) and on that of Chapter 2 (a species-level analysis). As taxa included in this study did not encompass all genera represented in the morphological studies, the matrix of morphological characters used here was condensed to include only taxa that were sequenced.

A total of 122 morphological characters were used in this study, including gross morphological characters of adults and larvae and chaetal characteristics. Opercular brood chambers were recoded as separate characters (*i.e*, those of the Januini and

Pileolariini may not be homologous) to prevent any bias in the inference of their multiple or single origins. Both formalin-preserved and fresh specimens were studied when available. See Appendix 3-1 and 3-2 for the list of morphological characters and the character matrix respectively and Section 2.2.2 for a more detailed description of characters.

3.2.6 Phylogenetic analysis

Four data sets were analyzed: 18s alone, 28s alone, combined 18s+28s, and combined 18s+28s+morphology. The molecular data sets were subject to Maximum Parsimony (MP) and Maximum Likelihood (ML) analysis with PAUP* v.4 beta 10 (Swofford 2003); and Bayesian analyses with MrBayes v. 3.0 beta 4 (Huelsenbeck & Ronquist 2001). The 18s+28s+morphology data set was subject to MP analysis only. In both combined analyses, the sequences of two outgroup taxa were combined into a composite sequence in two cases: A sabellid sequence consists of *Sabella spallazanii* (18s; Genbank) and *Eudistylia vancouveri* (28s; this study) and the *Serpula* sequence consists of *S. vermicularis* (18s; Genbank) and *S. columbiana* (28s; this study). The composite sequences were constructed using GeneTool 2.0 by overlapping homologous regions. These composite sequences were also used in the 18s+28s+morphology data set. There were no conflicts in scores of morphological characters between the composite taxon pairs.

Maximum Parsimony Analyses - MP analyses were unweighted and performed with heuristic searches (random stepwise addition, TBR branch-swapping, multitrees in effect, with 10 trees held at each step). Bootstrap support was tested with 1000 replicate searches.

Maximum likelihood analyses - Modeltest v.3.06 (Posada & Crandall 1998) was used to determine the appropriate model of sequence evolution for the 18s, 28s and 18s+28s data sets. The Akaike Information Criterion (AIC) selected the GTR + I + Γ model of evolution for all three molecular data sets. This model and the given parameter estimates for each data set were used to obtain ML topologies in PAUP* (Heuristic searches, TBR, random addition, 1 tree held at each step).

Bayesian analyses - MrBayes v. 3.0 beta 4 (Huelsenbeck & Ronquist 2001) was used under the GTR + I + Γ model of evolution. All analyses were run with default priors (rate matrix: 0-100, branch lengths: 0-10, and gamma shape: 0-1), 4 Markov chains (three heated and one cold) and a random starting topology. For each data set, 5 analyses were run with 500,000 generations with a tree saved every 100 generations. For each run, the first 100,000 generations (1000 trees) were discarded as *burnin*. The resulting trees were pooled from the 5 analyses for a total of 20,000 trees. Their majority rule consensus tree yielded posterior clade probabilities. Parameter estimates and their 95% confidence limits were determined from these 20,000 values.

Decay analysis - Decay values (Bremer 1994) were used as an alternate measure of support (to MP bootstrapping) for the 18s+28s+morphology data set. These values were computed using Autodecay 4.0.2 (Ericksson 1998).

Assessing congruence of data sets - Following Bull *et al.* (1993), possible data incongruence was assessed using both ILD Tests (Farris *et al.* 1995) and SH Tests (Shimodaira & Hasegawa 1998) in PAUP*. All topologies tested were those from MP analysis. To detect effect of any one taxon on the topologies, both the ILD Test and the SH Tests (for both 18s and 28s data sets) were repeated with sequential deletion of taxa.

The ILD test was done using 1000 replicate heuristic searches (TBR, random addition sequence, 10 trees held at each step) for both the 18s+28s data set and the 18s+28s+morphology data set.

Four series of SH Tests were performed: 18s data fitted to (1) 28s trees and (2) morphology trees; and 28s data fitted to (3) 18s trees and (4) morphology trees. All parameter estimates for the RELL ML evaluation were those estimated in Modeltest.

Ancestral state reconstructions - To reconstruct the ancestral states of brooding modes, we used Mesquite 1.05 (Maddison & Maddison 2004) for ML reconstructions. Reconstructions were performed on a combined 18S+28S+morphology topology, as this data set was assumed to be the most reliable estimate of spirorbin polychaete phylogeny. ML reconstructions were done with the Stochar module (Maddison & Maddison 2004) with marginal probability reconstruction and the Mk-1 model (branch lengths set to equal). Decisions of significance were made at the threshold of 2.0 units difference in -ln(likelihood).

I used three different coding schemes for the ancestral state reconstructions: (1) 6 states – free spawning (hypothesized ancestral mode), 4 tube brood modes (gelatinous masses of the Circeini, posterior epithelial attachment of the Spirorbini, loose embryo string of the Paralaeospirini, and thoracic brood stalk of the Romanchellini), and opercular brooding as one state; (2) 7 states, opercular brood chamber (OBC) coded as two states (Pileolariini epithelial OBC and Januini cuticular OBC; *Amplicaria* OBC coded as pileolariin-type; Knight-Jones 1973), all other states the same as analysis 1; and (3) 8 states; opercular brooding coded as 3 states, assuming that the OBC of *Amplicaria* is unique, all other states the same as analysis 2.

3.3 RESULTS

3.3.1 18s sequence analysis

The 18s data set had 1585 unambiguously aligned characters (645 variable; 498 of which were parsimony informative). Unweighted MP analysis yielded 12 trees with a length of 1829 steps. These trees were consistent with the topologies inferred by the ML and Bayesian analyses (Fig. 3-1). ML estimates of model parameters, although comparable to those provided by Bayesian analyses, did not fall within the 95% confidence intervals except in one case (r_{AT}) (Table 3-3). Similarly, the estimates from the five Bayesian runs (differing in random starting topology) did not all converge on statistically similar likelihoods (Table 3-3). No single run contributed to this result, but instead significant differences among runs for all parameters were found in three to eight of 10 multiple comparisons (Fisher's post hoc, significance level <0.05). Thus to provide the most representative clade support and parameter estimates, these data were pooled across all five runs.

Table 3-3 GTR + I + gamma parameter estimates

Data set	Parameter	ML Estimate	Bayesian Estimate	95% Confidence Interval	Difference among runs (*P*)
18s	- ln *L*	11605.1934	11587.7018	11587.8059, 11587.5979	0.0001
	r_{CT}	3.5863	3.7119	3.7066, 3.7172	0.0001
	r_{CG}	0.8234	0.8195	0.8179, 0.8211	0.0001
	r_{AT}	1.3036	1.3157	1.3135, 1.3179	0.0001
	r_{AG}	2.0057	2.0818	2.0785, 2.0851	0.0001
	r_{AC}	1.0343	1.0675	1.0653, 1.0697	0.0001
	π_A	0.2501	0.2477	0.2475, 0.2479	0.0001
	π_C	0.2329	0.2326	0.2324, 0.2328	0.0001
	π_G	0.2788	0.2798	0.2796, 0.2800	0.0001
	π_T	0.2382	0.2398	0.2396, 0.2400	0.0001
	α	0.5724	0.5737	0.5725, 0.5749	0.0150
	pinvar	0.3140	0.2892	0.2886, 0.2898	0.0064
28s	ln *L*	10790.1504	10729.9358	10729.8370, 10730.0346	0.0409
	r_{CT}	3.4666	3.5543	3.5494, 3.5592	0.0005
	r_{CG}	0.5970	0.5834	0.5822, 0.5846	0.0001
	r_{AT}	1.4045	1.3982	1.3955, 1.4009	0.0001
	r_{AG}	1.7817	1.7718	1.7687, 1.7749	0.0001
	r_{AC}	0.6273	0.6270	0.6254, 0.6287	0.0001
	π_A	0.1846	0.1862	0.1860, 0.1864	0.0001
	π_C	0.2527	0.2513	0.2511, 0.2515	0.0001
	π_G	0.3231	0.3243	0.3241, 0.3245	0.0001
	π_T	0.2396	0.2382	0.2380, 0.2384	0.0001
	α	0.7842	0.7493	0.7480, 0.7509	0.0001
	pinvar	0.1902	0.1602	0.1596, 0.1608	0.0001
Combined	- ln *L*	22388.9551	22298.3043	22298.2053, 22298.4033	0.0001
18s+28s	r_{CT}	3.4335	3.4445	3.4405, 3.4484	0.0001
	r_{CG}	0.7413	0.6974	0.6964, 0.6984	0.0001
	r_{AT}	1.1968	1.1840	1.1826, 1.1854	0.0013
	r_{AG}	1.7142	1.7058	1.7038, 1.7078	0.0001
	r_{AC}	0.7351	0.7451	0.7439, 0.7463	0.0001
	π_A	0.2289	0.2264	0.2262, 0.2266	0.0001
	π_C	0.2388	0.2405	0.2397, 0.2413	0.0001
	π_G	0.2957	0.2978	0.2976, 0.2980	0.0001
	π_T	0.2365	0.2353	0.2545, 0.2561	0.0001
	α	0.6374	0.6224	0.6216, 0.6232	0.0453
	pinvar	0.2845	0.2678	0.2674, 0.2682	0.0004

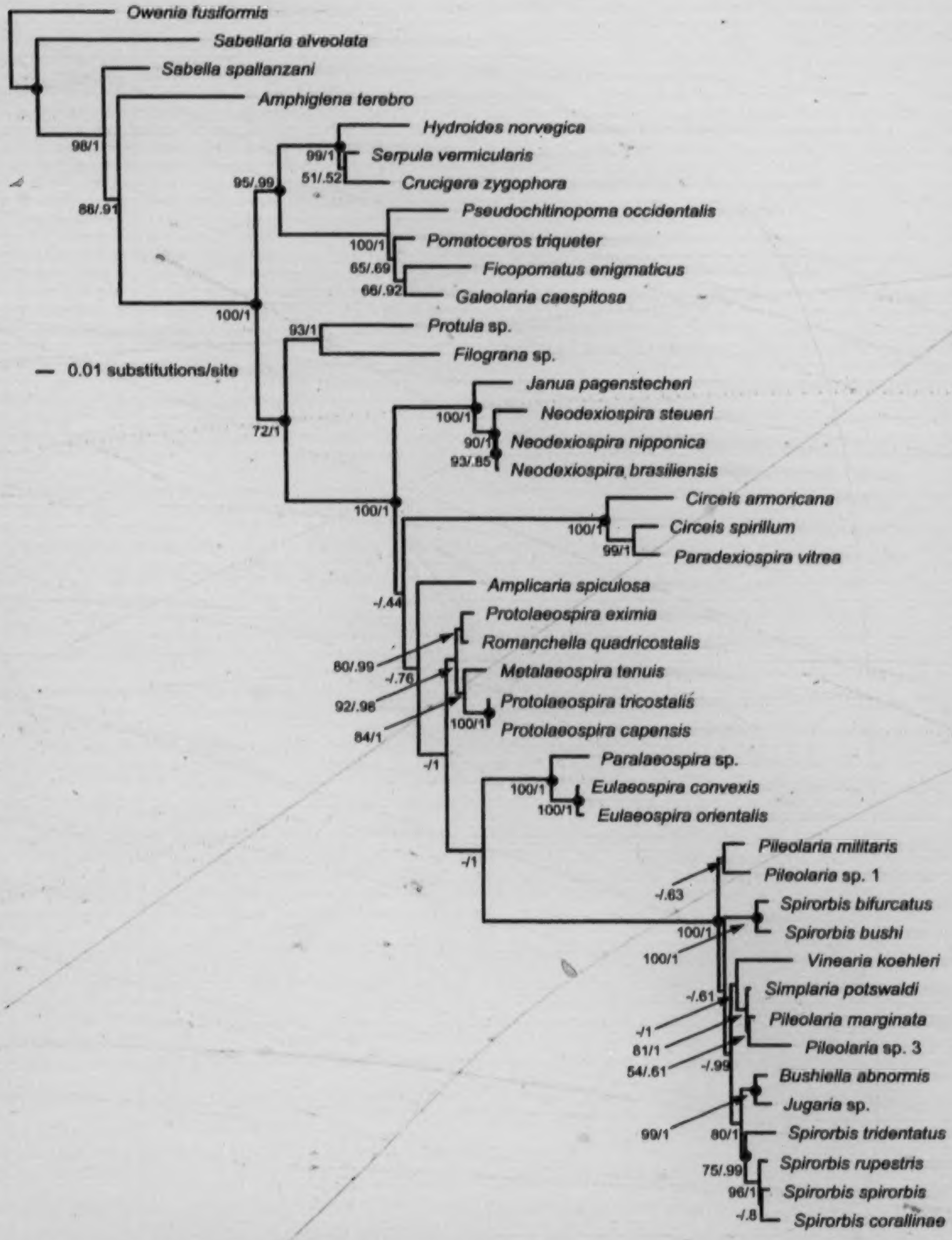
Owenia fusiformis
Sabellaria alveolata
Sabella spallanzani
Amphiglena terebro
Hydroides norvegica
Serpula vermicularis
Crucigera zygophora
Pseudochitinopoma occidentalis
Pomatoceros triqueter
Ficopomatus enigmaticus
Galeolaria caespitosa
Protula sp.
Filograna sp.
Janua pagenstecheri
Neodexiospira steueri
Neodexiospira nipponica
Neodexiospira brasiliensis
Circeis armoricana
Circeis spirillum
Paradexiospira vitrea
Amplicaria spiculosa
Protolaeospira eximia
Romanchella quadricostalis
Metalaeospira tenuis
Protolaeospira tricostalis
Protolaeospira capensis
Paralaeospira sp.
Eulaeospira convexis
Eulaeospira orientalis
Pileolaria militaris
Pileolaria sp. 1
Spirorbis bifurcatus
Spirorbis bushi
Vinearia koehleri
Simplaria potswaldi
Pileolaria marginata
Pileolaria sp. 3
Bushiella abnormis
Jugaria sp.
Spirorbis tridentatus
Spirorbis rupestris
Spirorbis spirorbis
Spirorbis corallinae
0.01 substitutions/site
98/1
88/.91
95/.99
99/1
51/.52
100/1
65/.69
66/.92
100/1
93/1
72/1
100/1
90/1
93/.85
100/1
100/1
99/1
-/.44
80/.99
-/.76
92/.98
84/1
100/1
-/1
100/1
100/1
-/1
-/.63
100/1
100/1
-/.61
-/1
81/1
54/.61
-/.99
99/1
80/1
75/.99
96/1
-/.8

Fig. 3-1 Maximum likelihood topology derived from 18s data set (-LnL=11534.965) with GTR+I+Γ model. Clade support is MP Bootstrap/Bayesian posterior probability below nodes. Dark circles indicate nodes shared by 28s rDNA ML topology.

Of the 40 nodes, 29 had posterior probabilities >0.95, and 27 had >70% bootstrap support. The nodes with low support tended to be either basal (higher order relationships among tribes) or separating extremely short branches (as in the Pileolariini + Spirorbini clade, Fig. 3-1). The 18s topology shares 15 nodes with that of the 28s topology (next section).

3.3.2 28s sequence analysis

The 28s data set inferred four MP trees of 2136 steps from the 952 included characters (582 variable; 446 parsimony informative). These topologies were congruent with those inferred by the ML and Bayesian analyses, which themselves inferred identical trees (Fig. 3-2). ML estimates of model parameters did not fall within the Bayesian 95% confidence limits with the exception of r_{AC} (Table 3-3). The 5 Bayesian runs produced similar -ln(likelihoods) (P= 0.0409, with only one run significantly different from the others (Fisher's post hoc test, P= 0.021; others all > 0.5)). However, they yielded different parameter estimates, and no single run was the cause (Fishers post hocs, 4-8 of 10 multiple comparisons significantly different for each parameter), and data were pooled for final values. Of the total 39 nodes in the ML tree, 25 had > 0.95 posterior probability and 23 had > 70% bootstrap support (Fig. 3-2). As with the 18s data, the lower support values were found in the most basal or recent splits. Fiteen nodes were shared with the 18s topology.

3.3.3 Morphology data analysis

MP analysis of the condensed morphological data set alone produced 47 trees of equal length (383 steps). These trees were mostly congruent with the previous hypotheses of Macdonald (2003; Section 2.3.2) with some notable exceptions: (1) Spirorbini were not basal, but were part of a derived clade with Pileolariini; (2) *Amplicaria spiculosa* occupied a more basal position on the tree and did not fall within Pileolariini; and (3) the Januini were basal and not a derived group within Pileolariini. Bootstrap support was low, with no basal splits supported with more than 50% bootstrap support. The morphological uniqueness of the Januini likely makes their placement on the tree somewhat labile.

3.3.4 Congruence among data sets

For the 18s+28s data set, the ILD test indicated that the partitions were incongruent ($P=0.01$), despite the topologies being similar (most significantly both infer a basal Januini and derived Pileolariini + Spirorbini). These results were confirmed by SH Tests on both ML and MP trees ($P< 0.001$ in both cases); for both 28s data optimized on 18s trees and 18s data optimized on 28s trees. Removal of any one taxon did not affect the conclusion of incongruence, using either test (ILD: $P< 0.05$ in all cases; SH: $P< 0.001$ in all cases; for both 18s and 28s data sets). For the 18s + 28s + morphology data set, the ILD test indicated no significant incongruence among the three partitions ($P= 0.14$). However, SH Tests detected significant incongruence when both the 18s and 28s data were mapped onto the morphology trees ($P< 0.05$ in all cases).

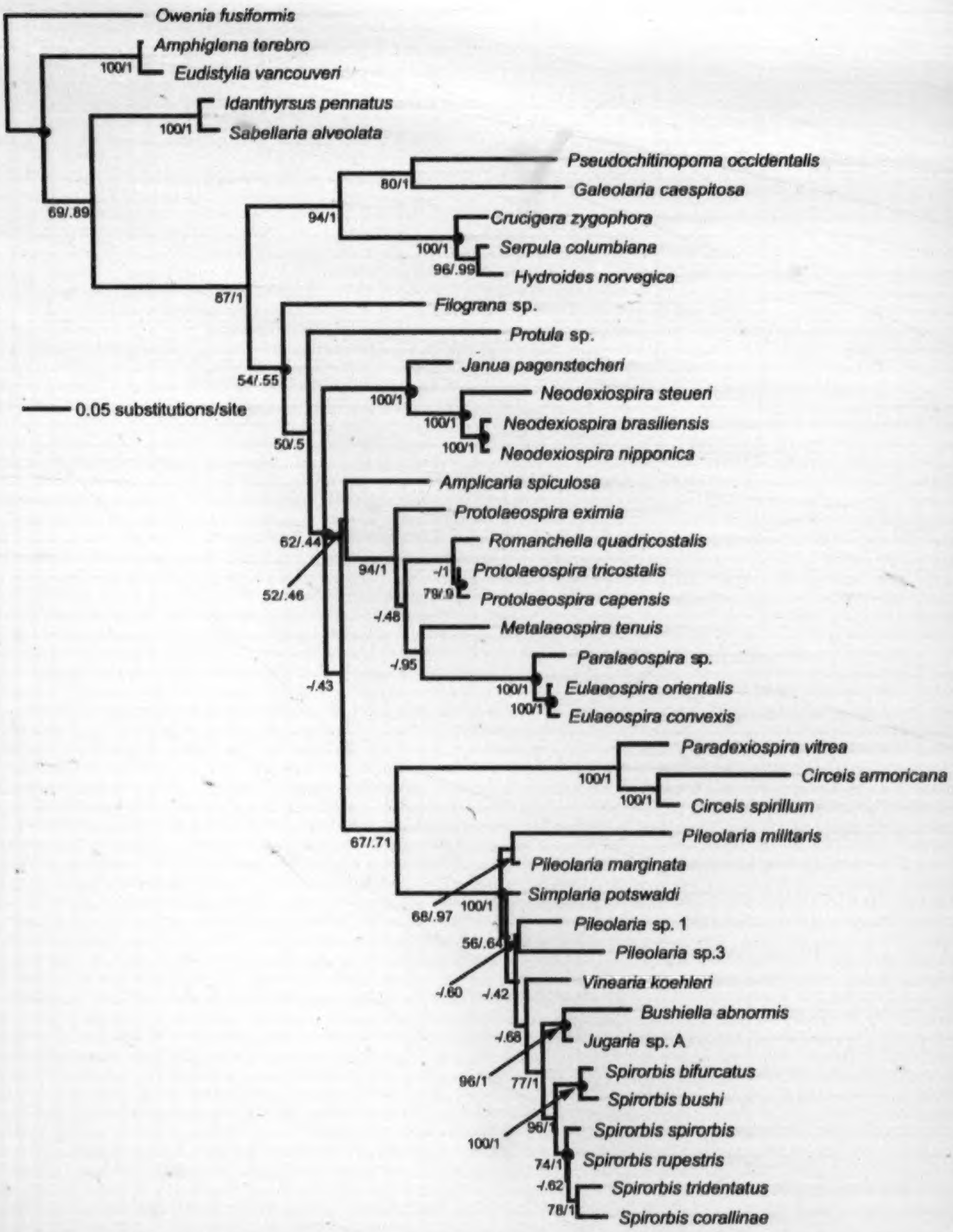
Owenia fusiformis
Amphiglena terebro
100/1
Eudistylia vancouveri
Idanthyrsus pennatus
100/1
Sabellaria alveolata
69/.89
Pseudochitinopoma occidentalis
80/1
Galeolaria caespitosa
94/1
Crucigera zygophora
100/1
Serpula columbiana
96/.99
Hydroides norvegica
87/1
Filograna sp.
Protula sp.
54/.55
Janua pagenstecheri
100/1
Neodexiospira steueri
0.05 substitutions/site
100/1
Neodexiospira brasiliensis
50/.5
100/1
Neodexiospira nipponica
Amplicaria spiculosa
Protolaeospira eximia
62/.44
Romanchella quadricostalis
94/1
-/1
Protolaeospira tricostalis
52/.46
79/.9
Protolaeospira capensis
-/.48
Metalaeospira tenuis
-/.95
Paralaeospira sp.
-/.43
100/1
Eulaeospira orientalis
100/1
Eulaeospira convexis
Paradexiospira vitrea
100/1
Circeis armoricana
100/1
Circeis spirillum
67/.71
Pileolaria militaris
Pileolaria marginata
Simplaria potswaldi
68/.97
100/1
Pileolaria sp. 1
56/.64
Pileolaria sp.3
Vinearia koehleri
-/.60
-/.42
Bushiella abnormis
-/.68
Jugaria sp. A
96/1
77/1
Spirorbis bifurcatus
Spirorbis bushi
100/1
96/1
Spirorbis spirorbis
74/1
Spirorbis rupestris
-/.62
Spirorbis tridentatus
78/1
Spirorbis corallinae

Fig. 3-2 ML topology derived from 28s data set with GTR+I+Γ model (-LnL=10681.83075). Clade support (below nodes) is MP Bootstrap/Bayesian posterior probability. Dark circles indicate nodes shared by 18s rDNA ML topology (Fig. 3-1).

Nonetheless, basal nodes are weakly supported in trees inferred from all three data sets (see Figs. 3-1 and 3-2 for 18s and 28s data sets), thus it was difficult to discern whether this incongruence was truly a result of conflicting phylogenetic signal of the two genes or strictly a symptom of this lack of resolution. Thus the data were analyzed in combination in an effort to improve this resolution.

3.3.5 Combined 18s + 28s data analysis

MP analysis of 2537 characters (1217 variable; 936 informative) yielded two equally parsimonious trees of length 3244. These trees were in conflict with the ML topology (Fig. 3-3). Conflicts occurred in the *Amplicaria* + Romanchellini + Paralaeospirini clade and the Pileolariini + Spirorbini clade. ML estimates did not fall within the 95% confidence limits inferred by the Bayesian analaysis. The Bayesian topology was in conflict with the ML topology (in both the clades mentioned above) as well as with the MP topology, but to a lesser extent (conflict located only within the Pileolariini + Spirorbini clade). Of the 39 nodes, 23 had bootstrap support >70% and 23 had posterior probabilities >0.95. This topology shared 18 nodes with the 18s ML topology and 26 nodes with the 28s ML topology (Fig. 3-3).

3.3.6 Combined 18s + 28s + morphology data analysis

MP analysis of the 2659 characters (1321 variable and 1050 parsimony informative; morphology alone has 113 informative characters out of 122 total) resulted in ten most parsimonious trees with a length of 4363 steps (Fig. 3-4). As with other analyses, tribes were strongly monophyletic but basal nodes lacked resolution.

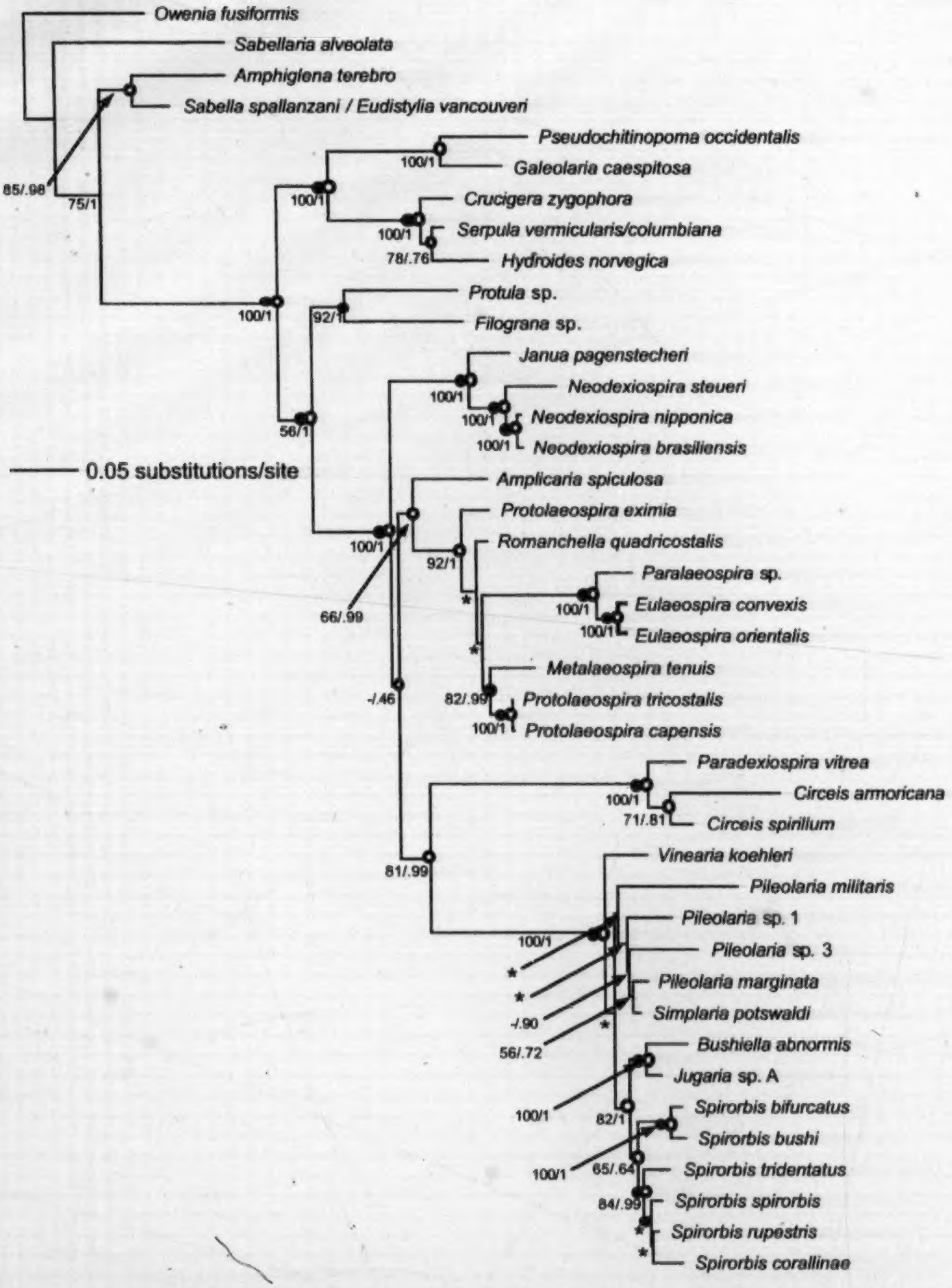
Owenia fusiformis
Sabellaria alveolata
Amphiglena terebro
Sabella spallanzani / Eudistylia vancouveri
85/.98
75/1
Pseudochitinopoma occidentalis
Galeolaria caespitosa
100/1
100/1
Crucigera zygophora
Serpula vermicularis/columbiana
100/1
78/.76
Hydroides norvegica
Protula sp.
100/1
92/1
Filograna sp.
Janua pagenstecheri
100/1
Neodexiospira steueri
100/1
Neodexiospira nipponica
100/1
Neodexiospira brasiliensis
56/1
0.05 substitutions/site
Amplicaria spiculosa
Protolaeospira eximia
100/1
Romanchella quadricostalis
92/1
Paralaeospira sp.
*
66/.99
100/1
Eulaeospira convexis
100/1
Eulaeospira orientalis
*
Metalaeospira tenuis
-/.46
82/.99
Protolaeospira tricostalis
100/1
Protolaeospira capensis
Paradexiospira vitrea
100/1
Circeis armoricana
71/.81
Circeis spirillum
Vinearia koehleri
81/.99
Pileolaria militaris
Pileolaria sp. 1
100/1
Pileolaria sp. 3
*
*
Pileolaria marginata
-/.90
Simplaria potswaldi
*
56/.72
Bushiella abnormis
Jugaria sp. A
100/1
Spirorbis bifurcatus
82/1
Spirorbis bushi
100/1
65/.64
Spirorbis tridentatus
84/.99
Spirorbis spirorbis
*
Spirorbis rupestris
*
Spirorbis corallinae

Fig. 3-3 ML topology from analysis of combined 18s+28s data sets with GTR+I+Γ model (-LnL=22250.1988). Clade support is MP bootstrap/Bayesian posterior probability. Asterisk denotes regions of incongruence of Bayesian and ML topologies. Black circles are nodes shared with 18s data ML topology, and open circles are nodes shared with 28s ML topology.

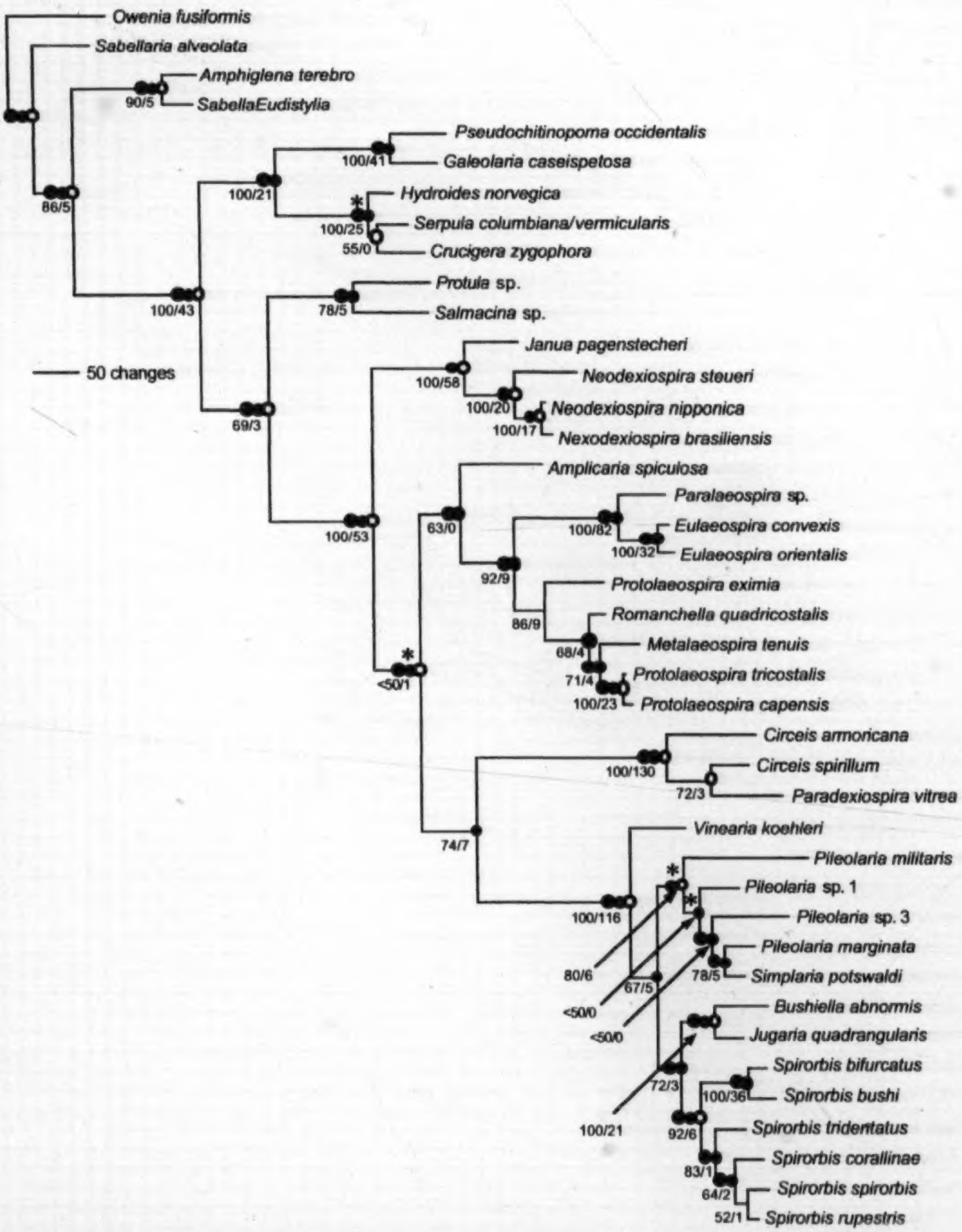
Owenia fusiformis
Sabellaria alveolata
Amphiglena terebro
90/5
SabellaEudistylia
86/5
Pseudochitinopoma occidentalis
100/41
Galeolaria caseispetosa
100/21
*
Hydroides norvegica
Serpula columbiana/vermicularis
100/25
55/0
Crucigera zygophora
100/43
Protula sp.
78/5
Salmacina sp.
50 changes
Janua pagenstecheri
100/58
Neodexiospira steueri
100/20
Neodexiospira nipponica
69/3
100/17
Nexodexiospira brasiliensis
Amplicaria spiculosa
Paralaeospira sp.
63/0
100/82
Eulaeospira convexis
100/53
100/32
Eulaeospira orientalis
92/9
Protolaeospira eximia
86/9
Romanchella quadricostalis
68/4
Metalaeospira tenuis
*
<50/1
71/4
Protolaeospira tricostalis
100/23
Protolaeospira capensis
Circeis armoricana
100/130
Circeis spirillum
72/3
Paradexiospira vitrea
Vinearia koehleri
74/7
Pileolaria militaris
*
Pileolaria sp. 1
*
100/116
Pileolaria sp. 3
Pileolaria marginata
80/6
67/5
78/5
Simplaria potswaldi
<50/0
Bushiella abnormis
<50/0
Jugaria quadrangularis
Spirorbis bifurcatus
72/3
100/36
Spirorbis bushi
100/21
92/6
Spirorbis tridentatus
Spirorbis corallinae
83/1
64/2
Spirorbis spirorbis
52/1
Spirorbis rupestris

Fig. 3-4 One of ten most parsimonious trees (haphazardly chosen) of tree length 4374 steps from combined analysis of 18s+28s+morphology data (asterisks denote nodes that collapse in strict consensus). Support values are MP bootstrap/Bayesian posterior probability. Black, open, and gray circles are nodes shared with 18s+28s Bayesian, 18s+28s ML. and morphology alone analyses respectively.

Differences among topologies lay within the Pileolariini + Spirorbini clade, and not among basal splits. Thus one of the ten topologies was chosen for ancestral state reconstructions, since my interest was in more basal splits with reference to the evolution of brooding modes and not in the more recent divergences.

3.3.7 Ancestral state reconstructions

Ancestral state reconstructions were done on the total evidence tree (combined 18s + 28s + morphology topology; Fig. 3-5). This tree was chosen because it was inferred from the most characters, and thus assumed to be the most reliable representation of spirorbin phylogeny (Bull *et al.* 1993; Kluge 1998). Both the 6- and 7-state reconstructions inferred opercular brood chambers (OBC) to be ancestral in Spirorbinae. Thus opercular brooding may have evolved only once at the base of the Spirorbinae clade and been lost among tube brooding tribes. The form of this ancestral brood chamber may be either a pileolarin-type or januin-type (both equally likely in the 7-state reconstruction). When *Amplicaria* was coded as a separate type of OBC (i.e., not as pileolariin-type), neither free-spawning nor opercular brooding could be reliably inferred as the ancestral reproductive mode.

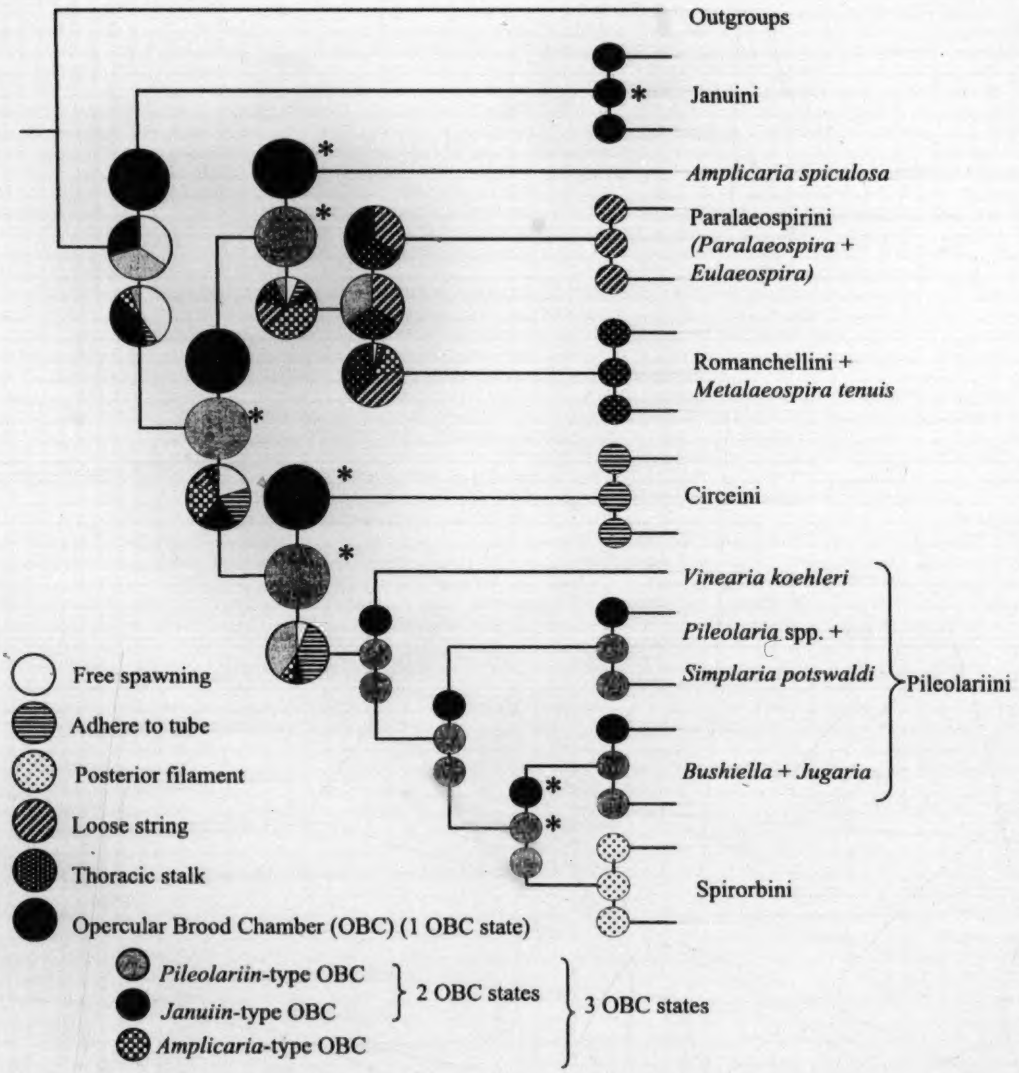
Outgroups
Januini
Amplicaria spiculosa
Paralaeospirini
(Paralaeospira +
Eulaeospira)
Romanchellini +
Metalaeospira tenuis
Circeini
Vinearia koehleri
Pileolaria spp. +
Simplaria potswaldi
Pileolariini
Bushiella + Jugaria
Spirorbini
Free spawning
Adhere to tube
Posterior filament
Loose string
Thoracic stalk
Opercular Brood Chamber (OBC) (1 OBC state)
Pileolariin-type OBC
Januiin-type OBC
Amplicaria-type OBC
2 OBC states
3 OBC states
*

Fig. 3-5 Simplified ML ancestral state reconstructions of brooding mode based on Mk-1 model and one of ten most parsimonious total evidence trees. Pie diagrams indicate likelihood of character states at that node; upper, middle and lower are those reconstructions with opercular brood chambers coded as one, two and three characters respectively. Only significant likelihoods are shown (-LnL≥2.0 units); asterisks denote pies with secondary non-signficant character-state likelihoods.

3.4 DISCUSSION

3.4.1 Incongruence among data sets

This discussion will focus on the phylogenetic relationships inferred by the 18s+28s+morphology trees for two reasons: (1) inclusion of different character types may avoid biases inherent in one data set (Bull *et al.* 1993), and (2) the topology inferred by the *most* characters may be the most accurate (Kluge 1998). We cannot discern if the incongruence among the three data sets reflects true genealogical discordance, or if the disagreement among trees is simply a reflection of their tenuous nature; these are poorly resolved nodes, and tests for incongruence (*e.g.*, ILD, SH) do not consider support for given nodes, and strictly compare topologies. Thus these tests are sensitive to giving false positives (Yoder *et al.* 2001; Shimodaira 2002). Incongruence that is not due to differing evolutionary histories is addressed by modifying the model used for phylogenetic reconstruction, and combining data is a way of dealing with such random topological differences that result from sampling error (Cunnningham 1997).

Considering the among-site rate variation of both the 18s gene (Abouheif *et al.* 1998) and the 28s gene (*e.g*, McArthur & Koop 1999, Colgan *et al.* 2000), partitioning 'by gene' could be considered arbitrary (Kluge 1998), as mutation rates within genes may differ just as much as among genes. The same argument can be applied to morphological data. So the data considered in combination allows more recent splits to be inferred, as well as the more basal ones inferred by the molecular data, as more resolution existed at the among-tribe level in the 18s+28s+morphology topologies than in trees inferred from molecular data alone (compare Figs. 3-3 and 3-4).

3.4.2 Phylogeny of the Spirorbinae

The data analyzed here support a monophyletic Spirorbinae. This relationship was strongly supported by the 18s only, 18s+28s, 18s+28s+morphology, and morphology only data sets (Figs. 3-1, 3-3 and 3-4, and Macdonald 2003 respectively), and weakly by the 28s data set, (Fig. 3-2). Surprisingly, the sister group to Spirorbinae is not Serpulinae, as hypothesized by Ten Hove (1984), Macdonald (2003) and Kupriyanova (2003) (based mostly on opercular morphology) but Filograninae, which have unmodified opercular stalks (*i.e.*, with pinnules), or lack opercula altogether, as in *Protula*. This relationship was not revealed by the morphological data alone and has been further investigated with molecular data by Kupriyanova *et al.* (2006) and Lehrke *et al.* (2006.).

All data sets infer strong support for a monophyletic Januini, Circeini, Pileolariini (excluding *Amplicaria*), and Spirorbini. Romanchellini + Paralaeospirini form a clade in most analyses, depending on the inclusion of *Metalaeospira* in Romanchellini (new position) or Paralaeospirini (traditional placement). Romanchellini and Paralaeospirini have been noted to share similar chaetal form and worldwide distribution (Knight-Jones & Knight-Jones 1984). They were found to form a clade by Macdonald (2003). In this study, these two tribes also group together, either as a clade (combined and 28s data sets) or as a grade (Romanchellini(Paralaeospirini(Pileolariini + Spirorbini))) (18s data set). Paralaeospirini, including only the genera *Paralaeospira* and *Eulaeospira* is monophyletic in all analyses (bootstrap support > 90%, pp= 1), but contains the third genus *Metalaeospira* only in the 28s data analyses (Fig. 3-2). Depending on its inferred position, Romachelliini (inclusive of *Romanchella, Protolaeospira*, and *Metalaeospira*) is either monophyletic (18s and 18s+28s+morphology) or rendered paraphyletic by containing Paralaeospirini (28s and18s+28s). All above relationships are supported with bootstrap >80% and posterior probabilities (pp) > 0.9 (or decay value = 9 in the case of the 18s+28s+morphology data set).

All data sets infer the sister group to other spirorbin tribes to be Januini, which was previously hypothesized to have a derived position (Macdonald 2003). This placement is inferred in both 18s and 28s data sets. Unfortunately the januin genera *Leodora* and *Pillaiospira* were not included in this study due to unavailability; but their inclusion would likely not affect the relationships of Januini to the remaining spirorbin tubeworms – they are distinct in both their morphology (Knight-Jones *et al.* 1975) and their molecular characters (sharing unique indels in both 18s and 28s sequences; Macdonald pers. obs). Their distinctive chaetation (*e.g.*, form of abdominal chaetae; Knight-Jones *et al.* 1975), larval morphology (two larval attachment glands; Höglund 1951), and opercular morphology (Thorp & Segrove 1975; Thorp 1975) do not provide clues about their relationship with other tribes, but reinforce their monophyletic status.

This dramatic shift in placement of Januini from that in Macdonald (2003) may result from: (1) their unique morphology (larval and adult) and corresponding lack of synapomorphies with other tribes making their placement on the tree somewhat labile (especially considering the original placement within Pileolariini was not well supported); and (2) the coding of reproductive characteristics as homologous characters (in particular opercular brooding). Other morphological characters they share with Pileolariini include the presence of larval attachment glands (paired in Januini, single in Pileolariini + Spirorbini) and the presence of paired crystalline thoracic patches in examined species. Upon reexamination of live material, this appears to be a coding error for the species examined here. Thus the preliminary hypothesis presented by Macdonald (2003) had less support than does the species-level morphological data analyzed here.

All data sets (including morphology) infer a derived Pileolariini + Spirorbini clade (exclusive of *A. spiculosa*). This grouping is supported by the presence of a single larval attachment gland and two symmetric thoracic tori (*e.g.*, Knight-Jones *et al.* 1979). Spirorbini appear to be a monophyletic group sister to *Bushiella* + *Jugaria* (bootstrap>70% and pp =1.00 for the 28s and combined data sets). The 18s data set infers a paraphyletic Spirorbini; but this is likely the result of the lack of resolution among the short branches in this clade. The combined data set infers a monophyletic *Pileolaria* spp. + *Simplaria*, sister to the *Bushiella*/*Jugaria* + Spirorbini clade. A basal member of this clade appears to be *Vinearia koehleri*; a relationship inferred by the combined data sets (albeit with moderate support; bootstrap < 70%, decay index = 5). However, both the 18s and 28s data alone place *Vinearia* within Pileolariini, but this topology lacks support (bootstrap< 50%, pp< 0.70). Interestingly, *Vinearia* has an operculum similar to that of *Amplicaria*: it is an open 'nest' instead of the typical enclosed brood chamber of the other pileolariins (Knight-Jones & Thorp 1984); but perhaps this similarity is convergent. Its possible basal position within Pileolariini challenges the view of Knight-Jones and Thorp (1984) that the opercular brood chamber of *Vinearia* is derived.

Despite being an opercular brooder, *Amplicaria* clearly is not a member of Pileolariini, as it groups with Romanchellini + Paralaeospirini in all analyses (except 18s alone, where it is more basal). These taxa tend to have an asymmetric distribution of thoracic tori (uncini-bearing segments) and chaetae (Knight-Jones 1973) and can be larger-bodied. *Amplicaria* shares these characters: it has five thoracic chaetigers on the convex side (four on the concave side), and has an asymmetric distribution of thoracic and abdominal chatae and uncini (Knight-Jones 1973). Also, both Romanchellini and Paralaeospirini release sperm clusters in eights or tetrads, as does *A. spiculosa* (G. Rouse pers. comm.). Most other tribes release sperm in clusters of many sperm. Romanchellini and Paralaeospirini also lack larval attachment glands; however, *A. spiculosa* may or may not have larval attachment glands, as it is never explicitly stated and we have yet to observe them. Thus an examination of brooding specimens is required, as is an investigation into the development and morphology of their OBCs.

The Circeini also lack larval attachment glands, and their position also remains unresolved. Inferred to be the sister group to Pileolariini + Spirorbini by the 28s (67% bootstrap support and 0.74 pp) and combined data sets (with >80% bootstrap and 0.99 pp), the 18s data set places them in a more basal position (sister to *Amplicaria* (Protolaeospirini(Paralaeospirini (Pileolariini + Spirorbini; however, bootstrap support <50% and pp = 0.44 pp). One notable characteristic they share with Pileolariini + Spirorbini is their thoracic 'crystalline patch', a ventral patch of granular pigment varying in shape and size among species (*e.g.,* Knight-Jones *et al.* 1979; pers. obs.). This patch tends to be more diffuse in Circeini and Spirorbini and may be paired in Pileolariini (Knight-Jones *et al.* 1979).

The phylogenetic affinity of Romanchellini and Paralaeospirini is clear from both our molecular data (28s and 18s rDNA) and morphological data (Macdonald 2003; Chapter 2). In fact, these two tribes are distinguishable only by the presence of romanchellin brush-like abdominal chaetae and their thoracic embryo attachment stalk, which is lacking in Paralaeospirini. However, this division has been blurry in the past. Both *Metalaeospira* and *Eulaeospira* were originally placed in Paralaeospirini (Knight-Jones 1973; maintained in Knight-Jones 1978). Knight-Jones & Knight-Jones (1994) placed *Metalaeospira* in Romanchellini (then Romanchellinae) when they observed that *M. armiger*, and possibly *M. pixelli*, had short brood stalks, which they described as 'oviduccal funnels'. A similar 'funnel' was observed in *M. clansmani,* resembling that of *Romanchella quadricostalis* (Knight-Jones *et al.* 1972). Additionally, the abdominal chaetae of *Metalaeospira* are brush-like like those of romanchelliins (Knight-Jones & Knight-Jones 1994). Our data support this placement of *Metalaeospira* in Romanchellini (Figs. 3-2, 3-3, 3-4, 3-5).

Knight-Jones & Knight-Jones (1994) also suggested that *Eulaeospira* be placed in the Romanchellini, as they possess brush-like abdominal chaetae. However, our results suggest this not appropriate as both species (*E. convexis* and *E. 'orientalis'*) group strongly with *Paralaeospira* in all analyses. They render the existing Romanchellini paraphyletic in the 28s and two combined analyses, occupying a derived position in this group. It appears there has been an evolutionary trend towards the loss of thoracic brood stalks, and towards the brooding of the embryo string loosely in the posterior faecal groove. The placement of *Metalaeospira* as sister to the *Eulaeospira* + *Paralaeospira* clade (28s data) indicates this genus may be an intermediate between the tribes (and the oviduccal funnel an intermediate character state); However, in the 18s and combined data sets, its placement with *Protolaeospira* suggests at least two losses of the thoracic brood stalk: In *Metalaeospira* and again in Paralaeospirini.

In Macdonald (2003) and Chapter 2, both *Metalaeospira* and *Eulaeospira* were coded as having thoracic attachment to the brood mass (of the 'oviduccal funnel' type) upon the suggestion of Knight-Jones & Knight-Jones (1994) that *Eulaeospira* may have a similar (as yet unobserved) attachment. However, both *Metalaeospira tenuis* and the two species of *Eulaeospira* examined in this study do not have such attachments, and so this must be considered a coding error. However, more *Metalaeospira* should be examined to study the transition from brood-stalk use to that of brooding freely in the tube. Interestingly, this transition appears to be associated with smaller brood size and smaller embryo size (pers. obs.).

Overall the results agree with known morphological synapomorphies for the various tribes, although the analyses lack good support for relationships among tribes. Nonetheless, the combined analysis does show some improved support over the preliminary hypothesis presented by Macdonald (2003). More sequence data and inclusion of some enigmatic taxa such as *Neomicrorbis*, *Crozetospira*, *Anomalorbis*, and *Helicosiphon* may shed some light on these higher-order relationships. Both reproductive and larval characteristics may be evolutionarily conserved, which should help focus future morphological studies of phylogenetic relationships within Spirorbinae.

3.4.3 Evolution of brooding modes

Contrary to previous hypotheses (*e.g.*, Macdonald 2003), the ancestral brooding mode is not tube incubation, but likely a form of opercular brooding (Fig. 3-5). However, the form of this ancestral opercular brood chamber (OBC) is unclear, as inferences depend on the coding of this character. When coded as one state (six-state reconstruction; Fig. 3-5), it is the most ancestral brooding mode and has clearly been lost in Romanchellini, Paralaeospirini, Circeini and Spirorbini. When *Amplicaria spiculosa* is coded as having an OBC homologous with the Pileolariini, the form of the ancestral brood chamber is not as clear; it is equally likely to have been similar to the OBC of the Januini or Pileolariini (seven-state reconstruction). Alternatively, when *A. spiculosa* is considered to have an OBC unlike the Pileolariini or Januini, ML reconstruction (eight-state; three opercular brooding states) indicates that a januin-type OBC is more likely to be the ancestral form than either the *Amplicaria*- or pileolariin-type.

The character coding of OBCs also affects conclusions about the number of times opercular brooding evolved; it could have arisen three times independently (once in Januini, once in Pileolariini, and once in *Amplicaria*-type spirorbins), or perhaps just once (and the different OBC forms are not a result of convergence but of homology). Further investigation into the development of the *Amplicaria* operculum is needed to determine which coding scheme is most justified. Knight-Jones & Thorp (1984) suggested *Amplicaria* had a basal opercular form, but did not investigate its development in detail. Thus this brood chamber could form by invagination of the opercular ampulla (as in Pileolariini), or by its swelling and subsequent degeneration (as in the Januini). Knight-Jones & Thorp's (1984) hypothesis of OBC evolution is based on adult morphology of representative species of Pileolariini and Januini only, and they concluded that the OBCs of the Januini were likely derived from those of the Pileolariini; a hypothesis we reject here, though supported by Macdonald's (2003) analysis. The *Amplicaria*-type OBCs are more likely derived from a januin-type ancestor. Knight-Jones & Thorp (1984) also suggested that the *Amplicaria* brood chamber is closely related to the 'open nest' brood chambers of *Vinearia* (represented here by *V. koehleri*) and *Nidificaria* (not included in this study). However, these two genera also possess typical characteristics of the Pileolariini (*e.g.*, two symmetric thoracic tori, dorsal thoracic crystalline patches) that *Amplicaria* does not share (Vine 1977; Knight-Jones 1973). Also, we do not know if *Amplicaria* possesses larval attachment glands (and how many), which would provide us with clear morphological evidence as to its tribal affinities considering the importance of this character in the evolution of the Spirorbinae. Its large body size and large number of thoracic tori (four on the convex side) supports its relatively basal position, and therefore we might expect it to have two larval attachment glands like the Januini. Alternatively, it may have none considering its apparent affinity with Romanchellini+Paralaeospirini.

Discussion of opecular brood chambers aside, both the six-state and eight-state ML reconstruction suggest 'free-spawning' is just as likely to be the ancestral form of reproduction among spirorbins as opercular brooding (Fig. 3-5). However, this possibility is not considered here; the sampling of Filograninae in this study (represented by *Protula* sp. and *Salmacina* sp.) is not sufficient to draw conclusions about which filogranin reproductive mode (of which there are many; Kupriyanova *et al.* 2001) is a precursor to those of Spirorbinae.

The greater diversity of opercular brooders compared to tube brooders has prompted speculation about the possible advantages to opercular brooding. To date, Hess (1993) has investigated the possibility that brood sizes of tube brooders are limited by allometric constraints and external opercular brooding represents a release from these constraints. She found no significant differences in scaling coefficients among tube and opercular brooders; indicating that limitations on brood size may not explain the relatively small numbers of tube-brooding species. However, no attempt was made by Hess (1993) to allow for non-independence of data based on phylogeny. Additionally, Hess's (1993) study presumed that opercular-brooding was derived from tube-brooding. The current study presents a different evolutionary hypothesis: if tube brooding did arise from opercular brooding independently at least three times (in Paraleospirini + Romanchellini, Spirorbini and Circeini), then perhaps the selective advantages of tube brooding modes have been overlooked. Tube brooding, rather than opercular brooding, may have multiple origins.

The hypothesis that the thoracic brood-stalk of Romanchellini is derived from a recessed radiole (Knight-Jones & Thorp 1984) and is a precursor to opercular brooding (*e.g.*, Knight-Jones *et al.* 1979; Macdonald, 2003) is not supported by the analysis here. The brood-stalk of the Romanchellini appears to be a derived condition. This hypothesis is supported by the assertion of Pillai (1960) that the romanchellin brood-stalk is not a likely precursor to an opecular brood chamber because it occupies a different position from the radioles of the opercular crown. The romanchellin brood-stalk appears to have arisen from the paralaeospirin-type brooding mode, *i.e.*, an embryo string is brooded loose within the tube with no attachment to adult structures. This finding contradicts Macdonald's (2003) hypothesis, which suggests the loose-string brooding was derived from species with a thoracic brood-stalk. Thus, contrary to our expectation, the brood-stalk of the Romanchellini might represent an elaboration of the 'oviduccal funnel' possessed by *Metalaeospira* (Knight-Jones & Knight-Jones 1994).

These results highlight the need for a reversal in polarity of previous hypotheses regarding the evolution of Spirorbinae. Also, they clearly illustrate the need for robust phylogenetic hypotheses in the discussion of life history evolution. The patterns observed here can better direct us in our studies of comparative life history traits and morphological adaptations of Spirorbinae.

3.4.4 Rapid radiation or a loss of phylogenetic signal?

This study shares the same fate as many molecular phylogenetic studies of invertebrates in general, and polychaetes in particular: lack of resolution among basal branches. This is a common occurrence in recent higher-order 18s phylogenies (*e.g.*, Bleidorn *et al.* 2003; Hall *et al.* 2004), and can be interpreted as either a rapid radiation event among annelids, or a fundamental limitation of 18s to resolve divergences of more than 500 million years ago (McHugh 2000). These hypotheses cannot be distinguished, as both result in a shortage of shared derived characters for inferring deep splits. However, spirorbin polychaetes may be old enough to reach the limits of 18s (and 28s) resolution: there are records of fossil impressions of tiny (<0.5 mm) coiled tubes from the Ordovician (Ruedemann 1934) that may be spirorbin tubeworms. This has recently been challenged by Taylor & Vinn (2006), who suggest these fossils belong to tiny microconchidian lophophorates.

Perhaps a more conserved gene may clarify the branching events at unresolved regions of the tree. Nevertheless, this study provides new insights into relationships and life history evolution in Spirorbinae, and as such presents opportunities for future morphological investigations - in particular, the comparison of opercular brood chamber morphology amongst a wider sampling of taxa, and the comparison of reproductive traits that may help explain observed phylogenetic patterns.

3.4.5 Future studies

Relationships observed in these analyses inspire further investigation. For example, the sister group relationship of the Filograninae to the Spirorbinae [instead of Serpulinae, as hypothesized by Ten Hove (1984)] was unexpected, and begs confirmation. This confirmation is provided by Lehrke *et al.* (2006) and Kupriyanova *et al.* (2006). The placement of *Amplicaria spiculosa* is also contentious, as it does not group as expected within the Pileolariini. Given that opercular brooding is likely ancestral, an understanding of the morphology and development of the brood chamber of *A. spiculosa* will play a pivotal role in our understanding of the evolution of opercular brooding.

Further investigation into phylogenetic relationships among the Spirorbinae with molecular data would be welcome. Most beneficial would be the inclusion of genera not represented here: *Nidificaria* (Pileolariini), *Pillaiospira* (Januini), *Leodora* (Januini), *Velorbis* (Spirorbini), *Helicosiphon* (Romanchellini) and more examples of *Paralaeospira* (Paralaeospirini). This might improve resolution of basal splits and elucidate transitions among brooding modes.

Also of benefit would be the addition of more molecular data. In particular, a more conserved marker to discern relationships among tribes. This might be elongation factor 1-α (*e.g.*, McHugh 1997), or perhaps amino acid sequences or gene arrangements (although it is unclear if this would be informative at the species level; Boore & Brown

2000). Improving on the existing analyses will not only clarify the evolution of brooding modes, but also questions of broader evolutionary significance: the evolution of tube coiling, its directional asymmetry, and its relationship to miniaturization.

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Appendix 3-1 List of characters used in morphology data set

1 Chaetal inversion; 0: absent, 1: present
2 Body divided into thorax and abdomen; 0: absent, 1: present
3 Protstomium; 0: reduced, 1: branchial crown
4 Palps; 0: peristomial, 1: prostomial
5 Peristomial collar; 0: absent, 1: present
6 Nuchal organs; 0: absent, 1: present
7 Buccal organ; 0: absent, 1: present
8 Calcareous tube; 0: absent, 1: present
9 Coiled tube; 0: absent, 1: present
10 Coiling direction; 0: dextral, 1: sinistral
11 Calcareous tube type; 0: chalky, 1: porcellanous; 2: vitreous
12 Longitudinal ridges; 0: absent, 1: present
13 Growth rings; 0: absent, 1: present
14 Peripheral flange; 0: absent, 1: present
15 Crystalline patch; 0: absent, 1: present
16 Form of crystalline patch; 0: absent, 1: present, 2: paired, 3: single, 4: diffuse
17 Sexuality pattern; 0: simultaneous hermaphrodite, 1: gonorchorism
18 Egg production; 0: whole abdomen, 1: first 2-3 Ab segs
19 Sperm production; 0: whole abdomen, 1: posterior abdomen
20 Spermatids; 0: eights, 1: cluster, 2: tetrads, 3: single
21 Sperm head; 0: spherical, 1: elongate
22 Larvae; 0: lecithotrophs, 1: planktotrophs
23 Embryo incubation; 0: absent, 1: present
24 Location of embryo incubation; 0: tube, 1: operculum
25 Tube incubation; 0: loose in tube, 1: unattached string, 2: gelatinous matrix, 3: posterior filament, 4: thoracic stalk
26 Opercular brood chamber; 0: cuticular cylinder; 1: epithelial
27 Dorsal convex collar flap; 0: absent, 1: present
28. Number of radioles; 0: <10, 1: >10
29 Larval attachment gland; 0: absent, 1: single, 2: paired
30 Position of larval attachment gland; 0: anterior, 1: posterior
31 Number of pairs of larval ocelli; 1: one, 2: two, 3: three
32 Number of ciliary bands on trochophore; 0: two; 1: three
33 Metatrochophore collar; trochophore
34 Collar development; 0: post-settlement; 1: pre-settlement
35 Branchial bud development; 0: post-settlement; 1: pre-settlement
36 Anal vesicle; 0: unpaired, 1: paired, 2: absent
37 Long-handled thoracic uncinal hooks; 0: absent, 1: present
38 Operculum; 0: absent, 1: present
39 Operculum development; 0: post-settlement, 1: pre-settlement
40 Opercular calcification; 0: absent, 1: present
41 Number of opercula; 0: one, 1: two 42. Opercula stacked; 0: absent; 1: present
43 Opercular shape; 0: cone, 1: funnel 2: globular
44 Opercular basal processes; 0: absent, 1: present
45 Opercular Radiole; 0: modified (without pinnules), 1: unmodified (with pinnules)
46 Location of operculum; 0: left (concave) side; 1: either side; 2: both sides (double)
47 Edge of operculum; 0: smooth, 1: crenulate
48 Opercular plate; 0: absent, 1: present
49 Orientation of opercular plate relative to tube mouth; 0: perpendicular, 1: oblique
50 Opercular plate spines; 0: absent, 1: present
51 Secondary opercular plate below embryos; 0: absent; 1: present
52 Primary operculum becomes brood chamber; 0: absent, 1: present
53 Opercular plates retained after molting; 0: absent, 1: present
54 Brood chamber-talon fusion; 0: absent; 1: present
55 Insertion of opercular stalk; 0: Between first and second radioles, 1: outside radioles
56 Primary opercular plate rim; 0: absent, 1: present
57 Secondary opercular plate rim; 0: absent, 1: present
58 Fibre connecting talon and tori; 0: absent, 1: present
59 Primary talon; 0: absent, 1: present
60 Secondary talon; 0: absent, 1: present
61 Primary talon type; 0: spatulate, 1: vestigial, 2: tooth
62 Terminal talon bifurcation; 0: absent, 1: present
63 Talon projection; 0: absent, 1: present
64 Primary talon location; 0: eccentric, 1: peripheral, 2: centric
65 Primary talon external; 0: absent, 1: present
66. Distal opercular plate calcified; 0: absent, 1: present

67 Basal fusion of radioles; 0: absent, 1: present
68 Thoracic membrane; 0: absent, 1: present
69 Collar margin fused; 0: absent, 1: present
70 Number of segments, concave side; 0: three, 1: four, 2: five, 3: >five, 4: two
71 Number of thoracic tori, concave side; 0: two, 1: three, 2: four, 3: >five
72 Number of segments, convex side; 0: three, 1: four, 2: five, 3: >five, 4: two
73 Number of thoracic tori, convex side; 0: two, 1: three, 2: four, 3: >five
74 Thoracic tori symmetry; 0: symmetric, 1: asymmetric
75 Collar chaetae type; 0: fin and blade, 1: limbate, 2: geniculate, 3: bayonet
76 Gap between fin and blade; 0: absent, 1: present
77 Collar chaetae form; 0: straight, 1: bent
78 Same collar chaetae form convex and concave side; 0: present, 1: absent
79 Form of blade teeth; 0: fine, 1: coarse
80 Form of fin teeth; 0: fine, 1: coarse
81. Collar chaetae cross-striations; 0: absent, 1: present
82 Collar chaetae cross striation distribution; 0: present both sides, 1: absent concave side, 2: absent both sides
83 Capillary collar chaetae; 0: absent, 1: present
84 Capillary collar chaetae distribution; 0: absent, 1: both sides, 2: convex only
85 Capillary chaetae in second thoracic fascicle; 0: absent, 1: present
86 Capillary chaetae in third thoracic fascicle; 0: absent, 1: present
87 Sickle chaetae in third thoracic fascicles; 0: absent, 1: present
88 Shape of sickle chaetae; 0: parallel-sided, 1: pennant-shaped
89 Size of the distal portion of sickle chaetae; 0: >.25, 1: small, 2: >.5
90 Collar chaetae distribution; 0: symmetric, 1: more on convex, 2: more on concave
91 Thoracic uncini distribution; 0: more on concave, 1: more on convex, 2: symmetric
92 Multiple rows thoracic uncinal teeth; 0: absent, 1: present
93 Maximum number oflongitudinal rows of uncinal teeth; 0: six, 1: fifteen, 2: two, 3: three, 4: four
94 Transverse uncini rows; 0: straight,1: diagonal
95 Thoracic uncini peg type; 0: blunt, 1: pointed, 2: multi-pronged
96 Thoracic uncinal peg shape; 0: gouge-shaped, 1: flat, 2: round
97 Thoracic peg lateral teeth; 0: absent, 1: present
98 Segment with minimum number of thoracic uncini; 0: terminal thoracic setiger, 1: 2nd convex, 2: 3rd convex
99 Asetigerous region; 0: absent, 1: present
100 Number of abdominal chaetigers; 0: 0-10, 1: 11-20, 2: 21-30, 3: 30+
101 Abdominal uncini on convex side; 0: absent, 1: present
102 location of largest abdominal tori; 0: anterior, 1: posterior, 2: even distribution, 3: middle
103 Abdominal uncini symmetry; 0: symmetric, 1: asymmetric
104 Abdominal uncini beginning on first convex chaetiger; 0: absent, 1: present
105 Abdominal uncinial tooth transverse rows; 0: straight, 1: diagonal
106 Multiple abdominal uncini tooth rows; 0: absent, 1: present
107 Number of multiple abdominal uncini rows; 0: <ten, 1: >ten
108 Abdominal uncinal peg; 0: flat, 1: gouge-shaped
109 Anterior abdominal uncinal peg end shape; 0: blunt, 1: scalloped, 2: flared, 3: multi-pronged, 4: indented
110 Abdominal chaetae type; 0: geniculate, 1: trumpet-shaped, 2: simple, 3: capillaries only
111 Geniculate abdominal chaetae type; 0: pennant-shaped, 1: parallel-sided, 2: brush-like
112 Paired abdominal chaetae; 0: absent, 1: present
113 Distribution of geniculate abdominal chaetae; 0: entire abdomen, 1: posterior, 2: anterior
114 Capillary abdominal chaetae; 0: absent, 1: present
115 Capillary abdominal chaetae anterior-posterior distribution; 0: posterior, 1: anterior, 2: entire abdomen
116 Capillary abdominal chaetae left/right distribution; 0: concave, 1: convex, 2: both sides
117 First abdominal chaetiger with chaetae on convex side; 0: first, 1: second, 2: third, 3: fifth, 4: ninth, 5: tenth
118 First abdominal chaetiger with chaetae on concave side; 0: absent, 1: first, 2: second, 3: third, 4: fourth, 5: fifth
119 Abdominal chatae teeth; 0: fine, 1: coarse
120 First abdominal chatae tooth size; 0: same as other teeth, 1: first two small, 2: larger than other teeth, 3: first tooth small
121 Abdominal chaetae heel projection; 0: absent, 1: present
122 Size of abdominal chaetae vs. collar chatae; 0: same, 1: larger, 2: smaller

Appendix 3-2 Matrix of morphological characters used in total evidence analysis.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
1 *Owenia fusiformis*	0	0	0	1	0	0	1	0	0	-	-	-	-	-	0	-	1	0	0	3	0	1	0	-	-	-	0	1	0	-	-	-	0	-	-	-	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 *Sabellaria alveolata*	1	1	0	0	0	1	0	0	0	-	-	-	-	-	0	-	1	0	0	3	0	1	0	-	-	-	0	1	0	-	1	1	0	-	-	2	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3 *Amphiglena terebro*	1	1	1	1	1	1	0	0	0	-	-	-	-	-	0	-	1	0	0	3	0	0	0	-	-	-	0	1	0	-	1	1	0	-	0	2	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4 *Sabella / Eudistylia* composite	1	1	1	1	1	1	0	0	0	-	-	-	-	-	0	-	1	0	0	3	0	0	0	-	-	-	0	1	0	-	1	1	0	-	0	2	1	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5 *Pseudochitinopoma occidentalis*	1	1	1	1	1	1	0	1	0	-	0	0	0	1	0	-	1	0	0	3	1	1	0	-	-	-	0	1	0	-	2	1	0	0	0	0	0	1	0	0	1	0	2	0	0	1	0	0	-	-	-	-	-	-	0	0	0	0	0	0
6 *Serpula* composite	1	1	1	1	1	1	0	1	0	-	0	0	0	0	0	-	1	0	0	3	0	1	0	-	-	-	0	1	0	-	2	1	0	0	0	0	0	1	0	0	2	0	1	1	0	1	1	0	-	-	-	-	-	-	0	-	-	0	0	0
7 *Hydroides norvegicus*	1	1	1	1	1	1	0	1	0	-	0	0	0	0	0	-	1	0	0	3	0	1	0	-	-	-	0	1	0	-	2	1	0	0	0	0	0	1	0	0	1	1	1	0	0	1	1	0	-	-	-	-	-	-	?	-	-	-	-	-
8 *Galeolaria caespitosa*	1	1	1	1	1	1	0	1	0	-	0	0	0	1	0	-	1	0	0	3	0	1	0	-	-	-	0	1	0	-	2	1	0	1	0	1	0	1	1	0	1	0	2	0	0	1	0	0	-	-	-	-	-	-	?	-	-	-	-	-
9 *Crucigera zygophora*	1	1	1	1	1	1	0	1	0	-	0	1	0	0	0	-	1	0	0	3	0	1	0	-	-	-	0	1	0	-	2	1	0	0	0	0	0	1	0	0	1	0	1	1	0	1	1	0	-	-	-	-	-	-	0	-	-	-	0	0
10 *Protula* sp.	1	1	1	1	1	1	0	1	0	-	0	0	0	0	0	-	1	0	0	3	0	0	0	-	-	-	0	0	0	-	2	1	0	0	0	1	0	1	0	0	1	0	2	0	1	1	0	0	-	-	-	-	-	-	0	-	-	-	-	-
11 *Salmacina* sp.	1	1	1	1	1	1	0	1	0	-	0	0	0	0	0	-	0	0	0	3	1	0	1	0	0	-	0	0	0	-	2	1	0	0	0	1	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-
12 *Circeis armoricana*	1	1	1	1	1	1	0	1	1	0	1	0	0	0	1	4	0	1	1	1	1	0	1	0	2	-	0	0	0	-	3	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	-	-	0	0	0	0	0	1	1	1
13 *Circeis spirillum*	1	1	1	1	1	1	0	1	1	0	1	0	1	0	1	4	0	1	1	1	1	0	1	0	2	-	0	0	0	-	3	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	-	-	0	0	0	0	0	0	1	1
14 *Paradexiospira vitrea*	1	1	1	1	1	1	0	1	1	0	2	1	0	0	1	4	0	1	1	1	1	0	1	0	2	-	0	0	0	-	3	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	-	-	0	0	0	0	0	0	0	0
15 *Janua pagenstecheri*	1	1	1	1	1	1	0	1	1	0	1	1	1	1	0	-	0	1	1	1	1	0	1	1	-	0	0	0	2	0	3	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	1	0
16 *Neodexiospira nipponica*	1	1	1	1	1	1	0	1	1	0	0	0	1	0	0	-	0	1	1	1	1	0	1	1	-	0	0	0	2	0	3	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	1	0
17 *Neodexiospira brasiliensis*	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	-	0	1	1	1	1	0	1	1	-	0	0	0	2	0	3	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	1	0
18 *Neodexiospira steueri*	1	1	1	1	1	1	0	1	1	0	0	1	1	0	0	-	0	1	1	1	1	0	1	1	-	0	0	0	2	0	3	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	1	0
19 *Amplicaria spiculosa*	1	1	1	1	1	1	0	1	1	1	0	1	0	0	0	-	0	1	1	2	1	0	1	1	-	0	0	0	?	?	?	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	1	1	1	0	0	0	1	1	0	0	0
20 *Paralaeospira* sp.	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	-	0	1	1	2	1	0	1	0	1	-	0	0	0	-	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	-	-	-	0	0	0	0	0	1	1
21 *Eulaeospira convexis*	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	-	0	1	1	0	1	0	1	0	1	-	0	0	0	-	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	-	-	-	0	0	0	0	0	0	0
22 *Eulaeospira* cf. *orientalis*	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	-	0	1	1	0	1	0	1	0	1	-	0	0	0	-	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	-	-	-	0	0	0	0	0	0	0
23 *Metalaeospira tenuis*	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	-	0	1	1	0	1	0	1	0	1	-	0	0	0	-	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	-	-	0	0	0	0	0	0	0	0
24 *Protolaeospira striata*	1	1	1	1	1	1	0	1	1	1	2	0	1	0	0	-	0	1	1	0	1	0	1	0	4	-	0	0	0	-	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	1	0	-	-	0	0	0	1	1	1	1	1
25 *Protolaeospira tricostalis*	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	-	0	1	1	0	1	0	1	0	4	-	0	0	0	-	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	1	0	-	-	0	0	0	0	0	1	1	1
26 *Protolaeospira capensis*	1	1	1	1	1	1	0	1	1	1	1	0	1	1	0	-	0	1	1	0	1	0	1	0	4	-	0	0	0	-	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	1	0	0	-	0	0	0	0	0	0	1	1
27 *Romanchella quadricostalis*	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	-	0	1	1	0	1	0	1	0	4	-	0	0	0	-	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	1	0	0	-	1	0	0	0	0	0	1	1
28 *Vinearia koehleri*	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	2	0	1	1	1	1	0	1	1	-	1	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	1	0	0	1	1	0	0	1	1	0	1	1
29 *Bushiella abnormis*	1	1	1	1	1	1	0	1	1	1	0	1	0	0	1	2	0	1	1	1	1	0	1	1	-	1	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	1	1
30 *Jugaria quadrangularis*	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	2	0	1	1	1	1	0	1	1	-	1	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	1	1	1	0
31 *Pileolaria marginata*	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	2	0	1	1	1	1	0	1	1	-	1	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
32 *Simplaria potswaldi*	1	1	1	1	1	1	0	1	1	1	0	0	0	0	1	2	0	1	1	1	1	0	1	1	-	1	1	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	1	0
33 *Pileolaria militaris*	1	1	1	1	1	1	0	1	1	1	0	0	1	0	1	3	0	1	1	1	1	0	1	1	-	1	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	0
34 *Pileolaria* sp. 3	1	1	1	1	1	1	0	1	1	1	0	0	1	0	1	3	0	1	1	1	1	0	1	1	-	1	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
35 *Pileolaria* sp. 1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	1	3	0	1	1	1	1	0	1	1	-	1	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	?	0	0	0	0	0	0	0	0	1	0
36 *Spirorbis bifurcatus*	1	1	1	1	1	1	0	1	1	0	0	1	0	1	1	4	0	1	1	1	1	0	1	0	3	-	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	-	-	0	-	0	0	0	0	1	1
37 *Spirorbis spirorbis*	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	4	0	1	1	1	1	0	1	0	3	-	1	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	-	-	0	-	0	0	0	0	1	1
38 *Spirorbis bushi*	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1	4	0	1	1	1	1	0	1	0	3	-	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	0	0	-	-	0	-	0	0	0	0	1	1
39 *Spirorbis rupestris*	1	1	1	1	1	1	0	1	1	0	1	0	1	0	1	4	0	1	1	1	1	0	1	0	3	-	1	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	1	0	-	-	0	-	0	0	0	0	1	1
40 *Spirorbis corallinae*	1	1	1	1	1	1	0	1	1	0	1	0	0	0	1	4	0	1	1	1	1	0	1	0	3	-	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	1	0	-	-	0	-	0	0	0	0	1	1
41 *Spirorbis tridentatus*	1	1	1	1	1	1	0	1	1	1	1	1	0	0	1	4	0	1	1	1	1	0	1	0	3	-	0	0	1	1	2	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	1	0	-	-	0	-	0	0	0	0	1	1

Appendix 3-2 cont'd.

	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122
1	-	-	-	-	-	0	0	0	-	4	0	4	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	0	3	1	0	0	1	-	-	-	-	-	3	-	1	-	1	2	2	1	1	-	-	-	-
2	-	-	-	-	-	0	-	0	-	3	3	3	5	0	-	-	-	-	-	-	0	-	-	-	0	0	0	-	-	0	2	0	-	0	0	1	0	0	0	3	1	0	0	1	0	1	0	0	0	2	-	1	-	1	0	2	1	1	0	0	0	2
3	-	-	-	-	-	0	0	0	-	3	3	3	5	0	-	-	-	-	-	-	0	-	-	-	0	0	0	-	-	0	2	1	3	0	0	1	0	0	0	3	1	0	0	1	0	1	0	0	0	2	-	1	-	1	0	2	1	1	0	0	0	2
4	-	-	-	-	-	0	0	0	-	3	3	3	5	0	-	-	-	-	-	-	0	-	-	-	0	0	0	-	-	0	2	1	3	0	0	1	0	0	0	3	1	0	0	1	0	1	0	0	0	2	-	1	-	1	0	2	1	1	0	0	0	2
5	-	-	-	-	-	0	0	1	0	3	3	3	5	0	0	1	0	0	0	1	0	-	0	-	0	0	1	-	-	0	2	0	-	-	0	1	0	0	0	3	1	0	0	1	0	1	0	0	0	0	-	1	0	1	0	2	1	1	0	0	0	2
6	-	-	-	-	-	0	1	1	0	3	3	3	5	0	3	1	0	0	0	1	0	-	0	-	0	0	0	-	-	0	2	0	-	0	0	1	0	0	0	3	1	0	0	1	0	1	0	0	0	1	-	1	-	1	0	2	1	1	0	0	0	2
7	-	-	-	-	-	0	0	1	0	3	3	3	5	0	0	1	0	0	0	1	0	-	0	-	0	0	0	-	-	0	2	0	-	-	0	1	0	0	0	3	1	0	0	1	0	1	0	0	0	2	-	1	-	1	0	2	1	1	0	0	0	2
8	-	-	-	-	-	0	0	1	0	3	3	3	5	0	3	-	0	0	0	-	0	-	0	-	0	0	0	-	-	0	2	0	-	-	0	1	0	0	0	3	1	0	0	1	0	1	0	0	0	1	-	1	-	1	0	2	1	1	0	0	0	2
9	-	-	-	-	-	0	1	1	0	3	3	3	5	0	3	1	0	0	0	1	0	-	0	-	0	0	0	-	-	0	2	0	-	-	0	1	0	0	0	3	1	0	0	1	0	1	0	0	0	1	-	1	-	1	0	2	1	1	1	0	1	2
10	-	-	-	-	-	0	0	1	0	3	3	3	5	0	1	-	0	0	0	-	0	-	0	-	0	0	0	-	-	0	2	0	-	-	0	1	0	0	0	3	1	0	0	1	0	1	0	0	0	0	-	1	0	0	-	-	1	1	0	0	0	2
11	-	0	0	0	0	0	0	1	0	3	3	3	5	0	0	1	0	0	0	1	0	-	0	-	0	0	0	-	-	0	2	1	3	0	0	1	0	0	1	3	1	0	0	?	0	1	0	0	0	0	0	1	0	0	-	-	1	1	0	0	0	0
12	2	0	1	0	0	0	0	1	0	0	0	0	0	0	1	-	1	0	0	-	0	-	1	1	1	1	0	-	-	1	0	1	3	0	0	1	0	1	1	1	0	0	1	0	0	1	1	0	0	0	0	1	0	0	-	-	1	1	1	0	1	2
13	2	0	0	0	0	0	0	1	0	1	1	0	0	1	1	-	1	0	0	-	1	0	1	1	1	1	0	-	-	1	0	1	3	0	0	1	0	1	1	1	0	0	1	0	0	1	1	0	0	0	0	1	0	0	-	-	1	1	1	0	1	2
14	-	-	-	-	-	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	1	0	1	1	1	1	1	0	1	1	0	1	4	0	0	1	0	1	1	1	0	0	1	0	0	1	1	0	0	0	0	1	0	0	-	-	1	1	0	0	1	2
15	1	0	0	1	0	0	0	1	0	0	0	0	0	0	1	-	1	0	0	-	0	-	1	1	0	0	0	-	-	0	0	1	3	1	1	1	1	1	1	0	1	3	0	0	0	1	1	0	4	0	0	0	0	0	-	-	3	1	1	0	1	1
16	2	1	0	1	0	0	0	1	1	0	0	0	0	0	1	-	1	1	0	-	1	1	1	1	0	0	1	-	-	2	0	1	3	1	1	1	1	1	1	1	1	0	0	1	0	1	0	0	4	0	1	0	0	0	-	-	3	1	1	1	0	1
17	2	1	0	1	0	0	0	1	1	0	0	0	0	0	1	-	1	1	0	-	1	1	1	1	0	0	1	-	-	2	0	1	3	1	1	1	1	1	1	1	1	0	0	1	0	1	0	0	3	0	1	0	0	0	-	-	3	1	1	1	0	1
18	2	1	0	1	0	0	0	1	1	0	0	0	0	0	1	-	1	1	0	-	1	1	1	2	1	1	0	-	-	0	0	1	3	1	1	1	1	1	1	1	1	0	0	1	0	1	0	0	4	0	1	0	0	0	-	-	3	2	1	0	0	1
19	-	-	-	-	-	0	0	1	0	1	2	2	1	1	0	1	1	0	0	1	0	-	0	-	0	0	1	0	2	0	0	1	6	1	0	0	0	2	1	1	1	3	1	0	1	1	0	0	0	0	0	1	2	0	-	-	1	1	1	0	0	2
20	0	0	0	0	0	0	0	1	0	1	1	0	0	1	0	1	1	0	0	1	0	-	1	1	1	1	1	0	2	1	0	1	6	1	0	0	0	1	1	1	0	0	1	0	1	1	0	0	0	0	0	1	0	0	-	-	1	1	1	0	1	2
21	-	-	-	-	-	1	0	1	0	0	0	0	0	1	0	1	1	0	0	1	0	-	0	-	0	0	0	-	-	1	0	1	0	1	0	1	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	1	0	1	0	2	1	1	1	0	1	0
22	-	-	-	-	-	1	0	1	0	0	0	0	0	1	0	1	1	0	0	1	0	-	0	-	0	0	0	-	-	0	0	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1	0	0	4	2	-	1	-	1	2	2	1	2	-	-	-	2
23	-	-	-	-	-	1	0	1	0	1	1	0	0	1	1	-	1	0	0	-	0	-	1	1	0	1	1	0	0	0	0	1	0	1	0	2	0	1	1	1	1	2	1	0	1	1	0	0	4	0	2	1	0	1	0	2	1	2	1	3	1	0
24	0	0	1	0	0	0	0	1	0	1	1	0	0	1	0	1	1	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	0	1	0	0	0	2	1	2	1	0	1	0	1	1	0	0	0	0	2	1	0	0	-	-	5	1	1	2	1	2
25	0	0	0	0	0	0	0	1	0	1	1	0	0	1	0	1	1	0	1	1	1	0	1	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	2	1	0	1	0	0	6	1	1	2	1	2
26	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	1	1	0	1	1	1	0	0	-	0	0	1	0	0	0	0	0	1	1	0	1	0	2	1	1	0	0	1	1	1	1	0	0	0	0	2	1	0	1	0	0	7	4	1	0	1	2
27	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	-	1	0	0	-	0	-	0	-	0	0	1	0	0	0	0	1	4	1	0	1	0	1	1	1	1	0	1	0	1	1	0	0	0	0	0	1	0	1	0	2	1	1	1	2	1	1
28	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	1	0	1	1	0	-	1	1	0	1	1	0	2	0	0	0	0	1	0	1	0	2	1	1	1	2	1	0	1	0	-	0	0	0	0	1	0	0	-	-	6	2	1	3	0	2
29	2	0	1	1	1	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	-	1	1	1	1	1	1	2	0	0	1	1	1	0	0	0	2	1	2	1	3	1	0	1	0	-	0	0	0	0	0	0	1	0	2	1	1	1	0	1	2
30	2	0	0	1	1	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	-	1	1	1	1	1	1	0	0	0	1	6	1	0	0	0	2	1	1	1	3	1	1	1	0	-	0	0	0	0	1	0	1	0	2	1	1	1	0	1	2
31	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	0	0	1	0	2	0	0	0	-	-	0	0	0	1	1	1	1	1	0	1	1	0	-	1	0	0	0	1	1	1	0	2	1	2	1	0	1	2
32	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1	1	1	1	0	-	-	0	0	0	-	-	0	0	0	1	1	2	1	1	0	1	1	0	-	1	5	0	0	0	1	1	0	2	6	2	1	0	1	2
33	2	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	1	1	1	0	2	0	0	0	-	-	0	0	0	1	1	1	1	1	0	1	1	0	-	1	0	0	0	0	1	0	-	-	8	5	1	0	1	2
34	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	1	1	1	0	2	0	0	0	-	-	?	0	0	1	1	1	1	1	0	?	1	0	-	1	0	0	0	0	1	0	-	-	6	5	1	0	1	2
35	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	1	1	1	0	2	0	0	0	-	-	0	0	0	1	1	1	1	1	0	?	1	0	-	1	0	0	0	0	1	0	-	-	6	5	1	0	1	2
36	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	1	1	1	0	2	1	1	1	0	1	0	0	0	2	1	2	1	0	0	1	1	0	-	0	0	0	0	1	0	1	0	2	1	1	0	0	1	2
37	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	1	1	1	1	0	2	1	1	1	0	1	0	1	0	2	1	2	1	0	0	1	1	0	-	0	0	0	0	1	0	1	0	2	1	1	1	0	0	2
38	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	1	1	0	1	2	0	0	1	0	2	1	1	1	0	0	0	0	0	2	1	2	1	3	0	1	1	0	-	0	0	0	0	1	0	1	0	2	6	1	1	2	1	2
39	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	1	0	1	1	1	1	1	0	2	1	1	1	0	1	0	1	0	2	1	2	1	0	0	1	1	0	-	0	0	0	0	1	0	1	0	2	6	1	1	0	1	2
40	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	1	0	1	1	0	0	1	0	2	1	1	1	0	1	0	0	0	2	1	2	1	0	0	1	1	0	-	0	0	0	0	1	0	1	0	2	6	1	1	0	1	2
41	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	2	1	1	1	0	1	0	0	0	2	1	2	1	0	0	1	1	0	-	0	0	0	0	1	0	1	0	2	6	1	1	0	1	2

CHAPTER 4.

COMPARATIVE STUDY OF LIFE HISTORY TRAITS AMONG SPIRORBIN POLYCHAETES WITH DIFFERENT MODES OF BROODING

4.1 INTRODUCTION

Spirorbin polychaetes (Annelida, Sabellida, Serpulidae) present a unique opportunity to test hypotheses regarding the evolution of life history traits. Their diversity of brooding modes seems at odds with their relatively homogeneous appearance: all have coiled, miniaturized calcareous tubes (<5mm in spiral diameter) and show few other distinctive morphological differences (Macdonald 2003, Knight-Jones & Fordy 1979, Pillai 1970). However, they exhibit a remarkable diversity of brooding modes, whose costs and benefits remain little understood. Comparative studies of their reproductive traits may help us understand the course of evolution in the group, and perhaps the nature of selection acting on these miniaturized tubeworms.

Spirorbin tubeworms have six known modes of brooding, by which they are divided into tribes (formerly subfamilies; Bailey 1969, Knight-Jones *et al.* 1972, Knight-Jones 1978). The Paralaeospirini brood embryos loosely within the tube, with no attachment to adult structures (LOOSE); Circeini broods are attached to the inner tube wall and each other in a gelatinous matrix (MATRIX); Spirorbini embryos are arranged in strings that are attached to the inside of the tube by a tissue filament (STRING); the

Romanchellini have a thoracic brood-stalk that is thought to be homologous with a recessed tentacle (Knight-Jones & Thorp 1984) (STALK); and the Pileolariini (OBC-REUSE) and Januini (OBC-SHED) brood in a modified operculum, or a plug-like tentacle, that extends out of the tube mouth. The opercular brood chambers (OBCs) of the Pileolariini and Januini are morphologically distinct, differing markedly in development and structure (Knight-Jones & Thorp 1984): the Pileolariini possess an OBC that forms by the invagination of the opercular ampulla, and therefore has a double epithelium and a pore for embryo release; the Januni's OBC is a cuticular cylinder that forms by the swelling and subsequent degeneration of the ampullar epithelium (leaving behind a cuticular wall that must be dehisced for the release of embryos).

Opercular brood chambers were assumed derived within the Spirorbinae, as they have been considered a recent development (Elsler 1907, Borg, 1917, Gravier, 1923, Potswald, 1968, Pillai 1970). However, phylogenetic reconstruction suggests that the Januini-type (OBC-SHED) opercular brooding is ancestral in the Spirorbinae (Chapters 2 and 3; Macdonald & Rouse *In review*) and opercular brooding in general likely evolved twice (once again in the Pilolariini; OBC-REUSE). Tube brooding was also inferred to have multiple origins. Thus the gradual evolution of increasing embryonic attachment to adult structures (with a basal Paralaeospirini and derived opercular brooders) hypothesized by Knight-Jones & Thorp (1984) and Macdonald (2003) may be illusory.

The assumption that opercular brood chambers were novel was partly based on their patterns of species diversity among tribes. The Pileolariini and Januini together encompass more than two-thirds of the extant described Spirorbinae (*e.g.*, Kupriyanova *et al.* 2001, Knight-Jones & Fordy 1979), thus opercular brooding appears to have some benefit. However, comparative studies of spirorbin reproduction are few, as most have focused on the biology of individual species (*e.g.*, Daly 1978a, 1978b, Potswald 1967, Potswald 1968). Various studies have shown spirorbin embryos are brooded for approximately 2.5-4 weeks (Daly 1978a&b), and that upon larval release - when they have 3 chaetigers and a collar posterior to the prototroch cilia (Nott 1973) - they settle within hours or days (*e.g.*, Knight-Jones 1951, 1953), and the brood is usually replaced within a few days (Daly 1978a, King *et al.* 1969). An adult worm may have up to 3-4 broods in a brooding season (Daly 1978a; pers. obs.). Although life history data exists for various spirorbin species (*e.g.*, King *et al.* (1969), Potswald.1967, 1968, Daly 1978a&b, Hess 1993), comparative studies of tube and opercular brooders are lacking.

Opercular brood chambers were also assumed to be novel because miniaturization, as seen in the Spirorbinae (their ancestors being large-bodied; Lehrke *et al.* 2006, Kupriyanova *et al.* 2006), is often associated with novel morphology (Hanken & Wake 1993). The observation that notably large-bodied spirorbin species (with a large number of thoracic chaetigers) tend to brood inside their tubes reinforced this assumption (*e.g.*, Knight-Jones & Fordy 1979, Pillai 1970). Thus opercular brood chambers were thought accrue some sort of reproductive advantage. However, the effects of body size on various reproductive characteristics among brooding modes remain relatively unknown. Hess (1993) investigated possible allometric constraints on brood size among five spirorbin species (compared to large-bodied broadcast spawning serpulids) but found no differences among them.

Other comparative studies among spirorbins are lacking. Thorp & Segrove (1975) suggest that embryos of opercular brooders are more susceptible to predation, which may have a negative impact on fecundity but did not test this hypothesis. Increased oxygenation of the brood in an opercular brood chamber may also result in faster development and therefore faster turnover of the brood (P. Knight-Jones pers. comm.). However, these ideas remain uninvestigated.

Many aspects of the costs and benefits of spirorbin brooding modes remain unknown. In this study, I compared basic reproductive characteristics that affect lifetime fecundity and could therefore shed light on patterns of diversification among spirorbin tribes. These characteristics are: brood size, embryo size, brooding duration (developmental time of brood, from the placement of fertilized eggs into the brood mass to larval release), time between broods (the time from larval release to placement of the next brood into the brood mass), length of reproductive season, and minimum brooding body size. I compared these traits among ten spirorbin species from all six tribes to encompass the full diversity of spirorbin brooding modes.

Species differences in reproductive traits may help us understand evolutionary transitions among spirorbin tribes. Our new understanding of the phylogenetic relationships among the Spirorbinae has left many questions; this study may help us understand these unexpected patterns (*e.g.*, a basal Januni (OBC-SHED), derived and independently evolved Pileolariini (OBC-REUSE), and multiple origins of tube brooding). Alternatively, reproductive traits may not vary among brooding modes, in which case we may have to reassess evolutionary significance of brooding structures in this group.

4.2 METHODS AND MATERIALS

4.2.1 Brood, body and embryo size

The relationship between brood size and body size was investigated in ten species of spirorbins representing all six tribes: *Circeis armoricana* Saint-Joseph 1894 and *Paradexiospira vitrea* (Fabricius, 1780) (both Circeini - MATRIX), *Spirorbis bifurcatus* Knight-Jones, 1978 (Spirorbini - STRING), *Eulaeospira convexis* (Wisely, 1963) (Paralaeospirini - LOOSE), *Protolaeospira eximia* (Bush, 1904) (Romanchellini - STALK), *Neodexiospira brasiliensis* (Januini – OBC-SHED), *Simplaria potswaldi* (Knight-Jones, 1978), *Bushiella abnormis* (Bush, 1904), *Pileolaria marginata* Knight-Jones. 1978 and *Jugaria quadrangularis* (Stimpson, 1854) (all four Pileolariini – OBC-REUSE). All species were collected in Barkley Sound, except *E. convexis*, which was collected at Encounter Bay, South Australia, on drift algae and studied at the South Australia Museum (Adelaide, South Australia).

Fresh specimens were removed from their tubes without damaging adults or the brood, and placed in 4% magnesium chloride to relax them. The total number of embryos was counted (brood size), and the brood saved for later measurement. The adults were mounted on slides with seawater and glycerol for measurement under a dissecting scope mounted with a camera lucida and digitizing tablet (precision of 20 dots per mm; 25-50× magnification). Six measures of body size were recorded: spiral diameter (maximum diameter spanning all whorls of the tube starting at the tube mouth), tube diameter (at the tube mouth), convex and concave body length (smoothed length from the tip of the abdomen to the anterior edge of the collar along body margins furthest from, and closest to, respectively, the axis of the tube spiral, and maximum and minimum operculum diameter (the greatest distance across the orthogonal opercular plate, and an axis perpendicular to this; the endpoints are well-defined by the edges of the calcareous plate). These data were collected in the summer and fall of 2002, with the exception of *E. convexis*, which was collected in May 2004 in South Australia. See Table 4-1 for a complete list of sample sizes for each species.

Embryo size was recorded using the same digitizing system described above using a compound microscope. Five embryos of each brood (or the entire brood if less than 5 individuals) were mounted in seawater and their maximum and minimum diameters were measured. These measurements were used to compute individual embryo volume ($4/3\pi a^2b$, where a=major diameter, b=minor diameter of a prolate spheroid), and this value was averaged for each brood over the five embryos. Total embryo volume per brood was computed by multiplying the mean individual embryo volume times the total number of embryos.

4.2.2 Scaling coefficients

Scaling coefficients (coefficients of allometry) of brood size in relation to different measures of body size were computed as the slope of the regression of the log-log plot (Vogel 1988). The slope of the regression line relating brood size (assuming number of embryos is proportional to volume) and linear measures of body size, when log-transformed, should have a value of 3. To accommodate error in both the X and Y variables, the least-squares regression slope (LS) was also converted to a reduced major axis (RMA) slope by dividing the LS slope by the correlation coefficient, *r*, if the least-squares regression was significant (Vogel 1988). These values were compared to the expected value of 3 by a one-sample t-test (Zar 1996). It is unclear whether the LS or RMA slope presents the most appropriate measure of scaling, given the proportion of variance in brood size (y-axis) versus body size (x-axis) is unknown; brood sizes may be highly variable for a given body size.

4.2.3 Minimum brooding body size, and size at 50% brooding

Both the minimum body size (spiral diameter or tube diameter) observed brooding and the size at which 50% of the population were brooding were used as alternate measures of the age of first reproduction. The minimum brooding body size was strictly one observation per species: the size of the smallest observed brooding specimen of that species. Although informative, this inference relies on a single observation for an estimate of the minimum brooding body size. Thus I also used size at which 50% of the population was found to be brooding as an independent estimate.

To estimate this value (size at 50% brooding), a cumulative percent brooding curve was constructed for two measures of body size: spiral diameter (0.2 mm increments) and tube diameter (0.05 mm increments) in six species (see Table 4-4 for species and sample sizes). These dimensions were the least variable estimates of body size and therefore the most reliable. The resulting cumulative frequency curves, which were sigmoid, were linearized by a probit transformation (*e.g.*, Armitage & Berry 1994) and the size at which the probit=5 (corresponding to 50% brooding) was estimated through simple linear regression (Zar 1996).

4.2.4 Time between broods and brooding duration

The time between broods and the brooding duration of four tube-brooders (*Protolaeospira eximia, Spirorbis bifurcatus*, *Circeis armoricana* and *Paradexiospira vitrea)* and three opercular brooders (*Neodexiospira brasiliensis*, *Pileolaria marginata* and *Simplaria potswaldi)* were determined in the laboratory and the field. For both studies, reproductive worms were monitored daily for one full reproductive cycle (approx. 1 month, starting at the release of a particular brood).

Worms were monitored in one of three ways: (1) for *N. brasiliensis* and *S. potswaldi*, individuals on their original substrate were marked and surrounding spirorbins removed for ease of observation; (2) *P. marginata* (which lives on kelp), *C. armoricana,* and *S. bifurcatus* larvae were settled on glass slides or plastic sheeting and raised on these substrates until a reproductive age (this enabled me to see inside the tube to determine if a brood was present); (3) *P. vitrea* and *P. eximia* adult tubes (both of which are glassy and the inside more easily observed) were removed from their original substrates and glued (Krazy Glue, Elmer's Products, Columbus, OH) to slides. No damage to the tube was incurred, and this removal was assumed to have no effect on the variables of interest.

The prepared substrates (whether original or slides) were placed in a seawater table with running seawater at 8-10° C and seasonally appropriate day lengths maintained by artificial lighting at Bamfield Marine Sciences Centre (BMSC), or placed in the intertidal zone (the foreshore of BMSC, Bamfield Inlet) in slide cases bolted onto the rocks, or in the case of the worms on their original substrate, in mesh boxes affixed to the rock with cable ties. These experiments were done during the peak reproductive seasons of all species between autumn 2002 and summer 2005. All experiments were monitored daily to record the duration of one brooding cycle: day of larval release, days between broods, and the day of second larval release.

4.2.5 Reproductive season

The duration of the reproductive season in the field of four tube brooders (*Protolaeospira eximia, Spirorbis bifurcatus, Paradexiospira vitrea* and *Circeis armoricana)* and two opercular brooders (*Neodexiospira brasiliensis* and *Simplaria potswaldi)* were recorded in winter-summer 2003 and summer-fall 2004. Every two weeks, 40 individuals of each species (with the exception of *P. eximia*, of which only 20 were collected), were removed from their tubes and scored for brood presence or absence.

4.3 RESULTS

4.3.1 Brood size and embryo size

Brood sizes were highly variable within species, and they did not differ significantly among brooding modes (Table 4-1). Most species had an average brood size of 10-25 embryos, with the exception of *P. eximia* (Romanchellini, STALK), which had an average brood size of 60±42.5 embryos (range 10-155), and *B. abnormis* (Pileolariini, OBC-REUSE), which had an average brood size of 52±26.1 embryos (range 9-72 embryos). The size (volume) of individual embryos was also relatively constant among species (approximately 1-1.5×10^6 μm^3) with the exception of *B. abnormis,* whose embryos were unusually small ($0.33 \pm 0.11 \times 10^6$ μm^3), and *S. potswaldi*, which had the largest embryos ($2.3 \pm 0.61 \times 10^6$ μm^3). Notably, these two species with the smallest and largest embryos, respectively, have the same brooding mode (OBC-REUSE). The relationships between brood size and embryo volume among species was negative (Fig. 4-1A), although not statistically significant ($p = 0.16$). Nonetheless, overall reproductive effort (total embryo volume/brood) did increase with body size (Fig. 4-1B), but this was also not statistically significant ($p = 0.15$). If members of the Pileolariini (OBC-REUSE) were excluded, this relationship (among tube brooders and *N. brasiliensis* (OBC-SHED) became significant (albeit inferred from 6 data points) whereas the Pileolariini appeared to have no relationship between reproductive effort and body size ($p = 0.01$; Fig. 4-1B)

Within species, egg size (embryo volume) was constant, and in no case was the intraspecific relationship between brood size and individual embryo volume significant ($p > 0.1$ for all species). The stage of development also had no bearing on individual embryo volume, and did not vary significantly among larval stages (one-way ANOVA, $p > 0.1$), presumably due to the presence of the relatively rigid embryonic capsule.

Table 4-1 Average brood size and embryo size (average volume of 5 embryos, averaged over N individuals) for 10 species of spirorbin polychaetes. Embryo volumes were similar among all ontogenetic stages ($p = 0.1$) therefore embryo volume was computed from all ages of embryos pre-release.

Species	Brood mode	N	Brood size			Embryo volume (× 10^6 μm^3) Mean±SD
			Mean ± SD	Min.	Max.	
C. armoricana	MATRIX	35	18.1±14.02	3	55	1.5±0.31
P. vitrea	MATRIX	18	25.0±15.51	3	74	1.7±0.80
S. bifurcatus	STRING	16	17.1±9.11	7	33	1.5±0.77
P. eximia	STALK	32	59.7±41.15	10	155	1.2±0.38
E. convexis	LOOSE	21	10.2±10.04	5	28	1.2±0.31
N. brasiliensis	OBC-SHED	16	12.3±6.65	3	25	1.6±0.76
S. potswaldi	OBC-REUSE	17	17.2±9.72	7	45	2.3±0.61
P. marginata	OBC-REUSE	29	18.8±12.34	5	38	1.4±0.33
B. abnormis	OBC-REUSE	14	52.0±26.15	9	72	0.3±0.11
J. quadrangularis	OBC-REUSE	21	10.1±10.11	5	31	1.0±0.33

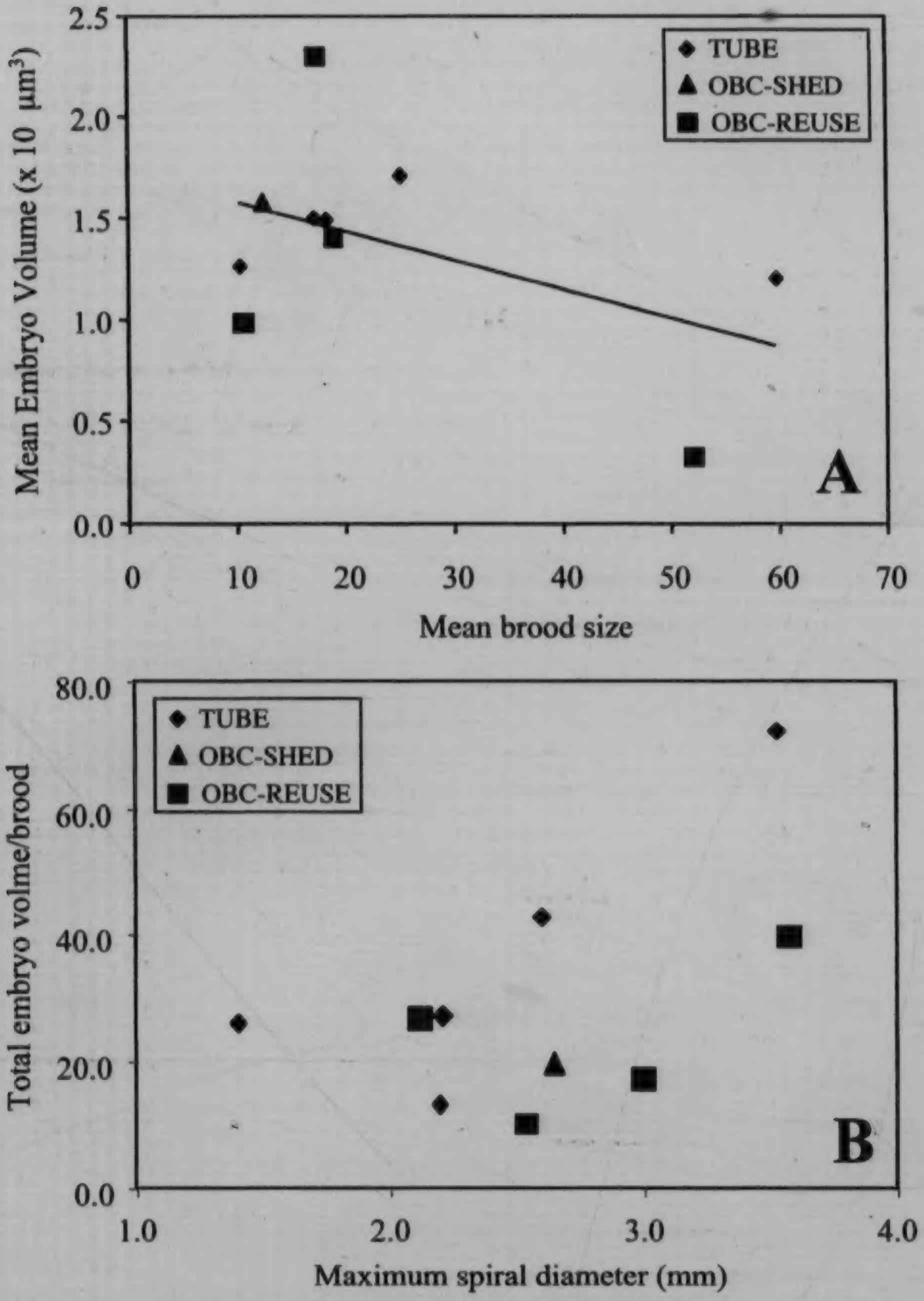

Fig. 4-1 Reproductive investment and size in ten species of spirorbin polychaetes. A. The relationship between individual embryo size and brood size (p= 0.16). **B**. The relationship of reproductive effort (total embryo volume/brood) and maximum adult body size (p=0.15). Large squares are members of the Pileolariini (OBC-REUSE), the triangle represents the Januini (OBC-SHED; one species) and the remainder are tube brooders.

4.3.2 Scaling coefficients

All species had a positive relationship between brood size and body size (Fig. 4-2). The slope of this relationship, or scaling coefficient, differed among species and measures of body size (Table 4-2). Eight species exhibited significant allometry of brood size (*i.e.*, slope of the log-log plot differed significantly from 3); the exceptions were *S. bifurcatus* and *N. brasiliensis*. Both positive and negative allometries were observed (more or less than 3 respectively) but the type of allometry depended more on the measure of body size than on any particular brooding mode. Significantly, the most extreme positive allometries (*P. marginata*, *J. quadrangularis*) and the most extreme negative allometries (*S. potswaldi, B. abnormis*) all occurred among species of the Pileolariini (OBC-REUSE; Table 4-2). Gelatinous matrix brooders (MATRIX) also exhibited significant positive (*C. armoricana)* and negative *(P. vitrea*) allometry.

Although there was no statistical evidence for consistent differences in allometry among brooding modes, it is notable that opercular brooder fecundity (brood size) seemed to depend less on body size: most Pileolariini (OPERC-REUSE) included here had a high proprortion of nonsiginificant LS regressions (Table 4-2; Figs. 4-2A&B) compared to tube brooders. The exception to this rule may be *S. bifurcatus* (STRING; Fig. 4-2A), which had no significant LS regressions for any measure of body size (Table 4-2).

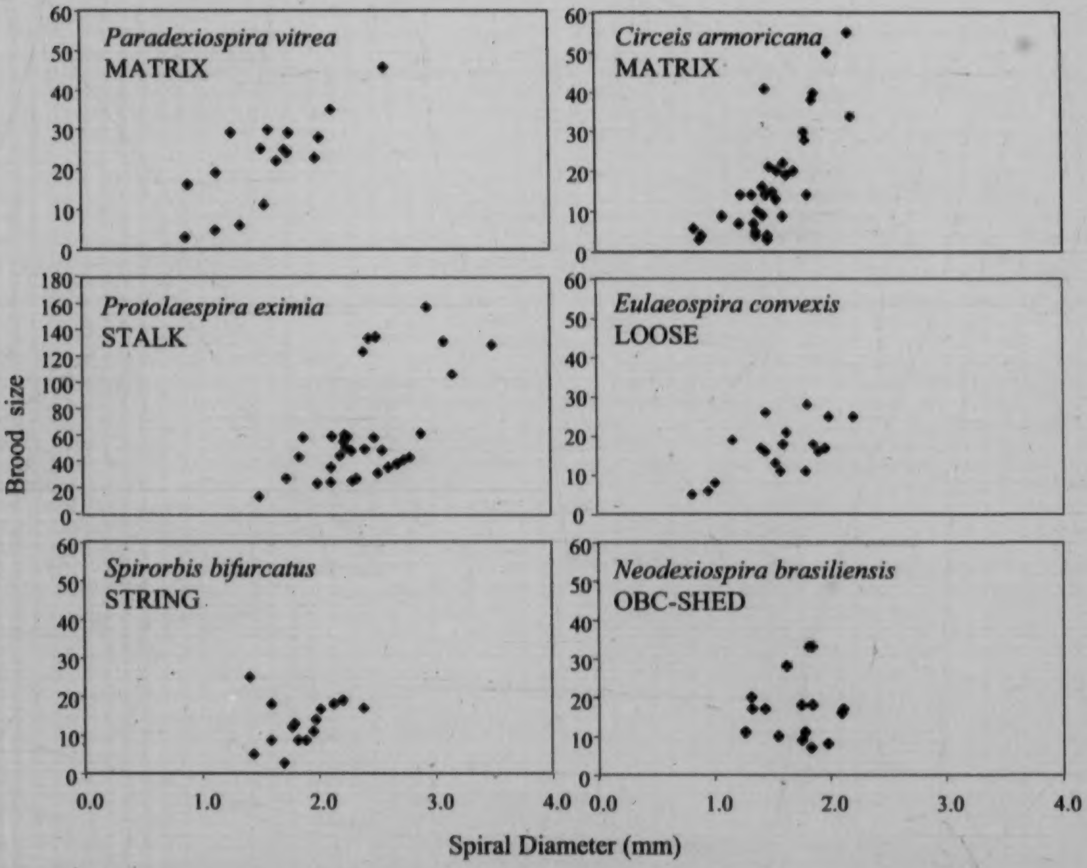

Fig. 4-2A Scatterplots of brood size (number of embryos) and body size (spiral diameter; the distance across all whorls from the tube mouth) for six spirorbin species with different brooding modes. All were from Barkley Sound, BC except *E. convexis* from Encounter Bay, South Australia. Note Y-axis scales are the same for allplots with the exception of *P. eximia*, which had very large brood sizes. Continued in Fig. 4-2B.

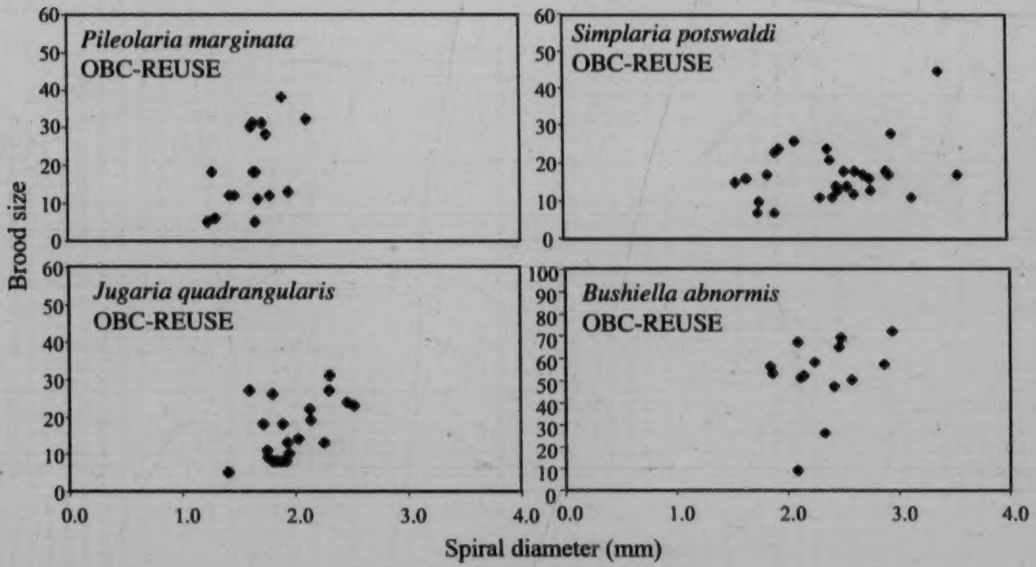

Fig. 4-2B Continued from Fig. 4-2A. Note all have the same Y-axis scale with the exception of *B. abnormis*, which had comparatively large brood sizes.

Table 4-2 Scaling coefficients for brood size and six linear measurements of body size of reproductive individuals of 10 species of Spirorbinae (con't on next page). LS- least squares linear regression slope, P – significance of LS, SE-standard error of LS, *r*-correlation coefficent of LS, RMA- reduced major axis slope, MEAN-mean trait value, SD-standard deviation of MEAN. Significant LS and deviations from isometry (expected value is 3) are in bold.

Species	Statistic	Tube Diam. (mm)	Spiral Diam. (mm)	Min. Opercular Diam. (mm)	Max. Opercular Diam. (mm)	Convex Body Length (mm)	Concave Body Length (mm)
C. armoricana	LS	**2.40**	**2.71**	**3.41**	**3.61**	**1.98**	**1.24**
MATRIX	P	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
N=35	SE	0.527	0.421	0.476	0.527	0.428	0.359
	r	0.622	0.747	0.781	0.766	0.627	0.516
	RMA	3.86	3.63	**4.37**	**4.71**	3.15	2.41
	MEAN	0.47	1.36	0.26	0.33	1.447	0.86
	SD	0.107	0.353	0.064	0.073	0.532	0.359
P.vitrea	LS	**1.72**	**2.00**	**1.77**	**2.33**	**1.77**	**1.45**
MATRIX	P	<0.001	<0.001	0.002	0.002	<0.001	0.001
N=18	SE	0.334	0.438	0.492	0.633	0.359	0.375
	r	0.816	0.752	0.671	0.678	0.776	0.694
	RMA	**2.11**	2.66	2.64	3.43	**2.28**	**2.08**
	MEAN	0.66	1.67	0.34	0.433	2.66	1.546
	SD	0.225	0.515	0.103	0.099	1.089	0.598
S. bifurcatus	LS	0.90	-0.07	-0.93	-1.60	0.20	0.44
STRING	P	0.187	0.931	0.105	0.071	0.713	0.344
N=16	SE	0.646	0.788	0.538	0.823	0.543	0.449
	r	0.348	0.024	0.420	0.463	0.100	0.253
	RMA	N/A	N/A	N/A	N/A	N/A	N/A
	MEAN	0.45	1.39	0.23	0.31	1.93	1.16
	SD	0.125	0.408	0.061	0.073	0.757	0.529
P. eximia	LS	0.27	**2.31**	**2.83**	**3.45**	**1.89**	**0.92**
STALK	P	0.731	<0.001	<0.001	<0.001	<0.001	0.052
N=32	SE	0.787	0.51	0.573	0.466	0.462	0.454
	r	0.064	0.638	0.669	0.803	0.599	0.347
	RMA	N/A	3.62	**4.23**	**4.29**	3.16	2.65
	MEAN	0.81	1.98	0.47	0.55	2.47	1.37
	SD	0.223	0.660	0.136	0.158	0.806	0.546
E .convexis	LS	**1.03**	**1.02**	**0.52**	**1.37**	**0.95**	**0.523**
LOOSE	P	0.018	0.052	<0.001	0.010	0.002	0.016
N=21	SE	0.393	0.483	0.337	0.465	0.247	0.194
	r	0.562	0.480	0.367	0.605	0.705	0.572
	RMA	1.83	2.13	**1.42**	2.26	**1.35**	**0.914**
	MEAN	0.46	1.33	0.23	0.32	1.61	1.07
	SD	0.135	0.457	0.086	0.095	0.767	0.593

Table 4-2 cont'd.

Species	Statistic	Tube Diam. (mm)	Spiral Diam. (mm)	Min. Opercular Diam. (mm)	Max. Opercular Diam. (mm)	Convex Body Length (mm)	Concave Body Length (mm)
N. brasiliensis	LS	**1.61**	1.67	-0.68	2.02	0.19	0.08
OBC-REUSE	P	0.040	0.082	0.490	0.328	0.833	0.903
N=16	SE	0.711	0.887	0.964	1.995	0.895	0.609
	r	0.518	0.448	0.186	0.261	0.057	0.033
	RMA	3.12	3.72	N/A	N/A	N/A	N/A
	MEAN	0.74	1.71	0.25	0.32	1.43	0.75
	SD	0.177	0.437	0.063	0.075	0.392	0.262
P. marginata	LS	1.21	**2.49**	**3.17**	1.87	0.62	0.24
OBC-REUSE	P	0.251	0.021	0.004	0.083	0.515	0.724
N=17	SE	1.031	0.956	0.930	1.005	0.923	0.652
	r	0.291	0.557	0.661	0.433	0.17	0.093
	RMA	N/A	4.46	**4.80**	4.32	N/A	N/A
	MEAN	0.65	1.56	0.29	0.36	1.74	0.92
	SD	0.131	0.289	0.057	0.068	0.399	0.255
S. potswaldi	LS	-0.36	0.46	0.48	0.32	**0.54**	**0.63**
OBC-REUSE	P	0.210	0.736	0.295	0.519	0.044	0.001
N=29	SE	0.282	0.324	0.453	0.5	0.254	0.179
	r	0.240	0.066	0.201	0.125	0.377	0.564
	RMA	N/A	N/A	N/A	N/A	**1.424**	**1.124**
	MEAN	0.71	1.80	0.32	0.41	1.98	1.163
	SD	0.274	0.741	0.108	0.124	0.831	0.572
B. abnormis	LS	1.68	0.94	**2.68**	1.48	**1.11**	**1.03**
OBC-REUSE	P	0.147	0.402	0.070	0.375	0.086	0.042
N=14	SE	1.087	1.084	1.347	1.602	0.596	0.454
	r	0.409	0.243	0.498	0.257	0.475	0.548
	RMA	N/A	N/A	**5.22**	N/A	2.34	**1.88**
	MEAN	0.53	1.80	0.34	0.44	1.90	1.07
	SD	0.175	0.648	0.128	0.133	0.773	0.475
J. quadrangularis	LS	**1.46**	**2.03**	**3.48**	**3.69**	**1.31**	**1.87**
OBC-REUSE	P	0.013	0.009	0.002	0.002	0.012	0.002
N=21	SE	0.535	0.694	0.995	0.998	0.472	0.524
	r	0.531	0.558	0.625	0.647	0.538	0.633
	RMA	2.75	3.64	**5.56**	**5.70**	2.44	2.95
	MEAN	0.57	1.79	0.35	0.40	1.40	0.99
	SD	0.134	0.367	0.092	0.075	0.417	0.221

4.3.3 Minimum brooding body size & size at 50% brooding

The minimum body size at which a species brooded tended to be larger in opercular brooders (approx. 1.2-1.8 mm spiral diameter; tube-brooders were all approx. 0.8 mm) (with the exception of the large-bodied *P. eximia*, which apparently started brooding at approx. 1.5 mm) (Table 4-3). But when tube diameter was used as a measure of body size instead of spiral diameter, this pattern was not evident.

The size at which 50% of individuals were brooding did not differ markedly between tube and opercular brooders (Table 4-3). Nonetheless, tube-brooding species tended to have the smallest values and opercular brooders tended to have the largest, thus there was a slight trend for opercular brooders to be larger at first reproduction (and presumably delayed in comparison to tube brooders). The exception to this was *P. eximia* (tube brooder; STALK), which had the largest body size with both measures. Both minimum brooding body size and size at 50% brooding increased with increasing body size (Fig. 4-3; p= 0.02 and 0.002 respectively, and r^2= 0.51 and 0.73 respectively).

4.3.4 Brooding duration and time between broods

The time between broods (mean number of days to replace a brood) differed significantly both among species and between locations (laboratory and field) (two-way ANOVA, $p< 0.0001$ in both cases). The interaction between these terms was also significant ($p< 0.001$). Multiple comparisons (Tukey's HSD) revealed nine species pairs (of a total 21 pairings) to be significantly different ($p< 0.001$ to $p< 0.01$), of which eight were between a tube and opercular brooder. These differences mainly resulted from a short time between broods in pileolariin species *P. marginata* and *S. potswaldi* (1.3-1.4

days and 2-2.1 days respectively; OBC-REUSE), and the longer time between broods of *N. brasiliensis* (4.2 and 6.5 days in field and lab respectively; tribe Januini, OBC-SHED). For all species except *S. potswaldi*, worms in the field replaced their broods faster than those in the laboratory (Table 4-4).

Brood duration, the mean number of days from the appearance of new embryos in the brood chamber until their release, differed significantly among species ($p< 0.001$) and between the laboratory and field ($p< 0.001$) two-way ANOVA). Their interaction was not significant ($p= 0.63$); the brood duration was always shorter in the field than in the lab (Table 4-4). Multiple comparisons revealed significant differences among almost all pairwise comparisons, thus the interspecific differences were distinct.

4.3.5 Reproductive seasons

Peak reproduction in all six spirorbin species occurred in the spring and summer (Fig. 4-4). Opercular brooders never exceeded more than 80% of the population being reproductive at one time (*N. brasiliensis*: Fig 4-5A, *S. potswaldi*: Fig. 4-5B). Tube brooders, however, often had more than 90% of examined individuals reproductive at one time (Figs. 4-5C- 4-5F; *C. armoricana*, *S. bifurcatus*, *P. vitrea* and *P. eximia*). The reproductive seasons of tube brooders also tended to be longer (at least March to November), whereas the two opercular brooders had more well-defined periods of non-brooding, and shorter reproductive seasons (May-November).

Table 4-3 Minimum brooding body size estimates for ten species of spirorbin polychaetes observed in the field. Shown are absolute smallest brooder observed, and the size at which 50% of individuals were brooding within the species, estimated by a probit transformation of the cumulative frequency curve using the spiral diameter and tube diameter (mm). Maximum brooding body size may be computed from the ratio of min/max.

Species	Brood mode	N	Minimum body size observed brooding (mm)		Size at 50% brooding (mm)	
			Spiral diam.	Tube diam.	Spiral diam.	Tube diam.
C. armoricana	MATRIX	61	0.83	0.30	1.32±0.434	0.46± .569
P. vitrea	MATRIX	50	0.89	0.26	1.39±0.156	0.52±1.009
S. bifurcatus	STRING	52	0.87	0.61	1.51±1.185	0.47±0.740
P. eximia	STALK	63	1.53	0.72	2.23±0.228	0.90±0.563
E. convexis	LOOSE	55	0.80	0.31	1.52±0.633	0.46±0.921
N. brasiliensis	OBC-SHED	33	1.38	0.48	1.61±0.738	0.67±1.076
S. potswaldi	OBC-REUSE	72	1.39	0.44	2.11±0.304	0.85±0.362
P. marginata	OBC-REUSE	22	1.24	0.48	1.44±1.779	0.63±1.014
B. abnormis	OBC-REUSE	46	1.87	0.53	2.14±0.921	0.61±1.693
J. quadrangularis	OBC-REUSE	33	1.41	0.42	1.78±0.722	0.57± 0.828

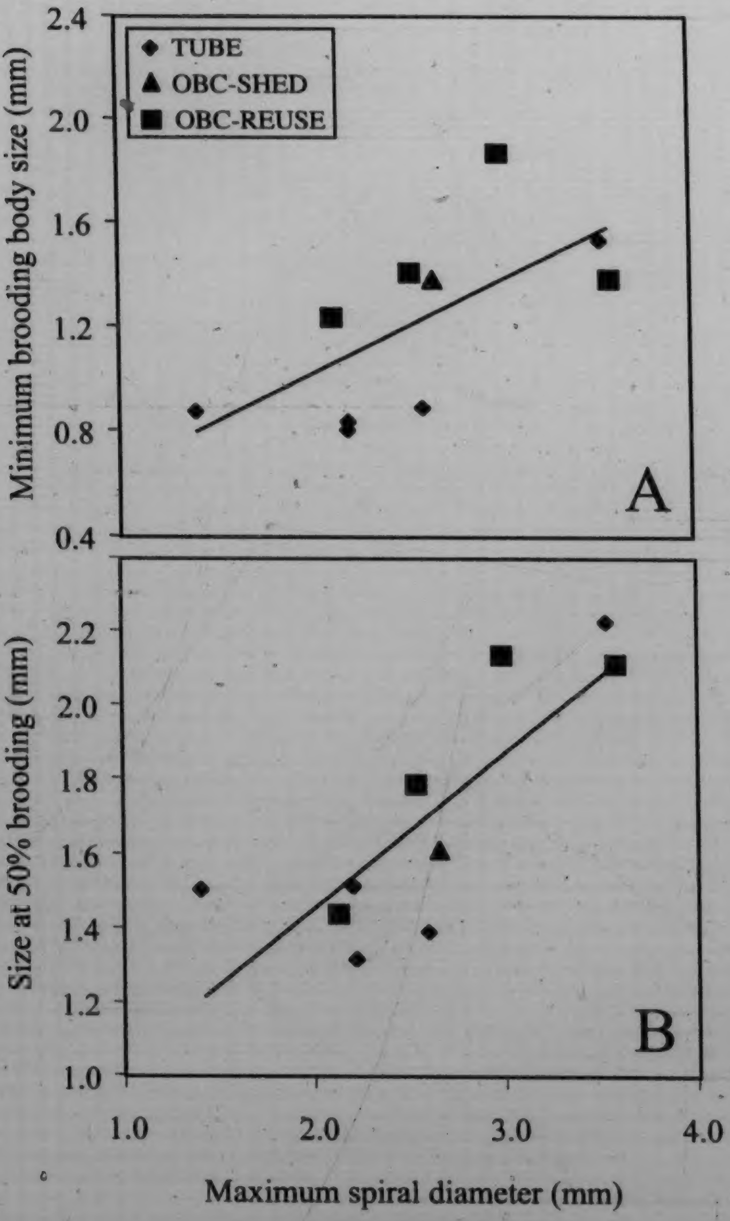

Fig. 4-3 The relationship of minimum brooding body size (**A**) and size at 50% brooding (**B**) to maximum body size (spiral diameter). Minimum body size needed to reproduce is not static but changes with the size of the adult worm. Large squares are Pileolariini (OBC-REUSE) and the triangle is Januini (OBC-SHED). All other data points are tube brooders.

Table 4-4 Time between broods and brooding duration of seven species of spirorbin polychaetes in Barkley Sound, BC, in the laboratory and in the field. Species include tube-brooders (*C. armoricana*, *P. vitrea*, *S. bifurcatus* and *P. eximia*) and opercular-brooders (*N. brasiliensis*, *S. potswaldi*, *P. marginata*).

Species	Brood mode	N		Brood duration (days) *a*		Time between broods (days) *b*		Brood turnover (days) [*a* + *b*]	
		Lab	Field	Lab	Field	Lab	Field	Lab	Field
C. armoricana	MATRIX	9	9	30.1±3.95	25.9±4.37	3.8±2.17	2.1±1.27	33.9±4.51	28.0±4.55
P. vitrea	MATRIX	9	10	33.3±2.38	31.3±2.38	3.0±2.45	2.9 ± 1.37	36.3±3.41	34.2±2.74
S. bifurcatus	STRING	10	9	30.4±4.39	27.1±2.47	3.2±1.87	1.7±0.87	33.6±4.77	28.8±2.62
P. eximia	STALK	7	6	31.6±1.72	27.8±2.32	6.3± 4.61	1.7±0.82	37.9±4.92	29.5±2.46
N. brasiliensis	OBC-SHED	8	9	27.9±1.89	23.3±2.92	6.1±1.81	4.2±1.64	34.0±2.61	27.6±3.35
S. potswaldi	OBC-REUSE	10	10	25.2±3.87	21.7±3.13	2.0±1.33	2.1±1.30	27.2±4.09	23.8±3.39
P. marginata	OBC-REUSE	10	10	23.0±2.00	16.9±2.23	1.4±0.52	1.3±0.68	24.4±2.07	18.2±2.33

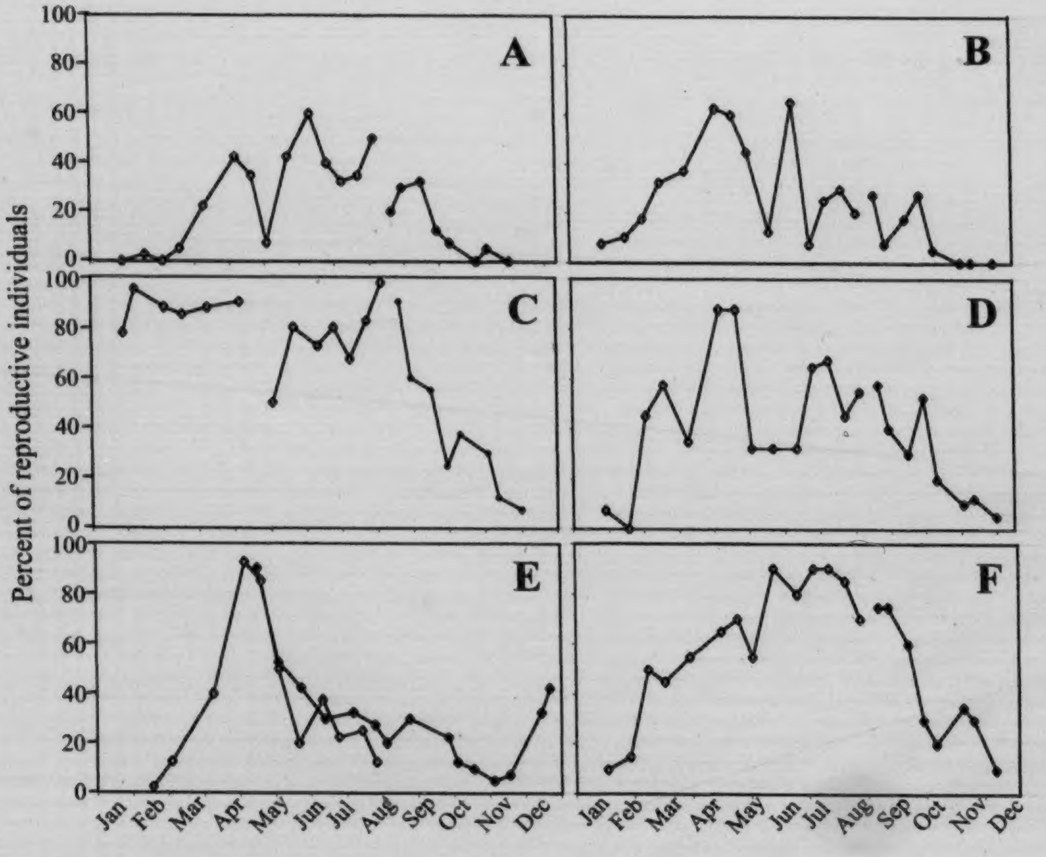

Fig.4-4 Reproductive seasons of six species of Spirorbinae. **A**. *Neodexiospira brasiliensis* (OBC-SHED), **B**. *Simplaria potswaldi* (OBC-REUSE), **C**. *Circeis armoricana* (MATRIX), **D**. *Spirorbis bifurcatus* (STRING), **E**. *Paradexiospira vitrea* (MATRIX) and **F**. *Protolaeospira eximia* (STALK). Open circles are data collected in 2004, and closed circles are from 2005. Percent reproductive individuals was based on 40 individuals collected in the field.

4.4 DISCUSSION

4.4.1 Major findings - differences among brooding modes

Studies of the tradeoffs among different modes of brooding in invertebrates are relatively few, as most comparative life history studies have focused on transitions between planktotrophy and lecithotrophy (*e.g.*, Fortunado 2004, Rouse & Fitzhugh 1994, Ponder & Lindberg 1997, Hadfield *et al.* 1995, Hart *et al.* 1997). Spirorbin polychaetes are unusual because they exhibit a diversity of brooding modes; thus they present a unique opportunity to investigate tradeoffs among different brooding strategies within a well-defined, monophyletic group (Pillai 1970, Macdonald 2003, Macdonald & Rouse *in review*).

Four striking differences among brooding modes were uncovered. First, with the notable exception of *P. eximia* (STALK), tube brooders tended to start brooding at smaller body sizes (Table 4-3). Second, in opercular brooders (+ *S. bifurcatus*) brood size was independent of body size (independent of the measure of body size), whereas body size in tube brooders (excepting *S. bifurcatus*) seemed closely tied with body size (Table 4-2). Third, the tube-brooding species studied here had a longer reproductive season, and over 75% of individuals in the population would be reproductive at one time (Fig. 4-4). The two opercular-brooding species studied here, on the other hand, had a more defined reproductive peak, *and* never exceeded ~75% of the population being reproductive at one time. This could have significant effects on overall fecundity if representative of opercular brooders. Finally, field data indicate brood duration and brood turnover was shorter for opercular brooders (<24 & <28 days respectively) than for tube brooders (this trend was not as apparent in the lab; Table 4-4).

Additionally, *Neodexiospira brasiliensis* (OBC-SHED), the only member of the Januini represented here, took longer to replace a brood than any other species (Table 4-4). This may result from the necessity of replacing the entire brood chamber after embryo release (Knight-Jones & Thorp 1984; in contrast to the Pileolariini, which can reuse their existing brood chamber). This could significantly affect lifetime fecundity, and warrants broader taxon sampling. It is possible *N. brasiliensis* is an exceptional case, given that it is close to the end of its range, as the Januini are more tropical in distribution and are most diverse in these areas (Knight-Jones & Knight-Jones 1984).

Nevertheless, lumping the Pileolariini and Januini together as 'opercular brooders' may be misleading given they may have different reproductive traits. This is not surprising, given their likely independent evolutionary origins (Chapters 2 & 3, Macdonald & Rouse *in review*).

To strengthen these observations, broader species sampling is required. We also need a more complete picture of all contributions to fecundity; for example, the number of broods per year, average lifespan, and growth rates may all contribute to overall fecundity. Both tube and opercular brooding species appear to have 3-4 broods/year (Daly 1978a and Potswald 1967 respectively), and similar lifespans (~1 year; Kupriyanova *et al.* 2001), but more comparative data would be useful.

4.4.2 Fecundity and body size

Most differences in reproductive traits among species observed here are attributable to differences in overall body size; however, reproductive output, allometry of brood size, and minimum brooding body size exhibited some differences among brooding modes.

Reproductive output - Brood sizes were highly variable among species (Table 4-1). This variability seems to be normal among many species of spirorbin tubeworms, given brood sizes reported in various other studies (*e.g.*, Daly 1978a, 1978b, Potswald 1967, 1968, Knight-Jones *et al.* 1979, Knight-Jones & Knight-Jones 1977). Explanations for this variability are not obvious, but could be related to success of fertilization or transfer of fertilized eggs to the reproductive structure. Small broods seem unlikely to result always from the lack of available resources, as spirorbin tubeworms thrive in conditions where they must only have bacteria to consume (Potswald 1967, 1968, pers. obs.) and still have large broods. However, the relative parental investment into the brood may depend on food quality as in other polychaetes (*e.g.*, Qian & Chia 1991).

Embryo size did not differ significantly among species (and therefore brooding modes), and differences that did exist may be explained by a corresponding difference in brood size (Fig. 4-1A). This negative relationship between brood size and embryo size is well established in invertebrates, although its causes and consequences remain relatively unexplored (Kolm *et al.* 2006, Hart 1993, Levitan 2000). Egg size is usually constrained within species, as this trait is canalized with very low variability (Wilkinson & Gibbons 2005). Spirorbin polychaetes do not, in this case, appear to be an exception. However, Daly (1978a) reports an increase in maximum oocyte diameter as the brooding season progresses, which may be related to the concurrent increase in the rate of oogenesis he observed with subsequent broods. Thus perhaps investigations into embryo size over the entire reproductive season are warranted, although this variability does not seem significant in comparison with large-scale differences among species.

Nevertheless, reproductive investment per brood generally increased with increasing body size (Fig. 4-1B), particularly among tube brooders (although p= 0.16 overall; p= 0.02 for tube brooders only). Thus overall body size influenced total reproductive investment per brood; but this effect was not as apparent in the Pileolariini (OBC-REUSE), as their reproductive investment per brood remained relatively constant with body size. Also, their brood sizes varied little with body size (Fig. 4-2). Investigations with more species, from more genera, are required to confirm these results and provide more independent contrasts of these intriguing patterns. It is surprising that only tube-brooding spirorbin tubeworms may gain the reproductive benefits of larger body sizes so well documented in other animals (Schmidt-Neilsen 1984, Schultz *et al.* 1991, Shine & Greer. 1991). This suggests there must be some other force driving body size evolution in spirorbins aside from fecundity.

Allometry of brood size - Overall, brood sizes did not consistently deviate from isometry, given that any significant deviations observed were not consistently negative or positive among the measures of body size used in this study (Table 4-2). However, some species did show weak overall tendencies towards negative allometry (*E. convexis* (LOOSE), *S. potswaldi* (OBC-REUSE) or positive allometry (*J. quadrangularis* (OBC-

REUSE, *P. eximia* (STALK), *C. armoricana* (MATRIX). Two species, *N. brasiliensis* (OBC-SHED), and *S. bifurcatus* (STRING) did not have significant relationships between brood size and body size (Fig. 4-2A, Table 4-2).

Minimum brooding body size – Tube brooders – with the exception of the large *P. eximia* - start brooding at smaller body sizes than opercular brooders (>1 mm and <1 mm respectively; Table 4-3) and therefore are likely to start brooding earlier, assuming they have similar growth rates. Why this minimum brooding size threshold is greater in opercular brooders is open to speculation: they may need to develop a certain degree of musculature to manipulate a large brood chamber. In addition, the development of the brood chamber itself could delay reproduction enough to affect fecundity, considering that both the Pileolariini and the Januini require the primary operculum to be replaced (Knight-Jones & Thorp 1984). This may be especially true for the Januini, which need to replace their brood chamber after each brood. The longer brood turnover time for *N. brasiliensis* observed here (Table 4-3) supports this idea.

When tube diameter was used as a measure of body size, the same pattern was not apparent; however, fluted tube apertures (e.g., *P. eximia, S. potswaldi*) may have reduced the value of this trait as a measure of body size among species. Nevertheless, further investigation into this phenomenon is warranted, especially using other measures of body size. Also, comparison of more species is needed, given Daly's (1978a) report that tube-brooding *Spirorbis spirorbis* broods at spiral diameters of greater than 1 mm, unlike the tube-brooders studied here.

Both minimum brooding body size and size at 50% brooding increased with increasing maximum body size (spiral diameter) (Figs. 4-4A and B respectively). Thus a threshold reproductive size is not fixed among species, but can be larger in extremely large-bodied species such as *P. eximia*.

4.4.3 Patterns of diversification

Opercular brooders are much more speciose than tube-brooding tribes (Knight-Jones & Fordy 1979). The Pileolariini (OBC-REUSE) alone encompass more than ~40% of described spirorbin species, and the Januini (OBC-SHED) ~30% (Kupriyanova *et al.* 2001), which suggests opercular brooding yields some sort of advantage. However, the persistence of diverse brooding modes in the Spirorbinae alone is evidence they all have some selective advantage.

The view of 'opercular brooders' as a group may be misleading, as the Januini and Pileolariini diverged long before the evolution of tube brooders (Chapters 2 & 3). The diversity of the Januini may simply be a result of their greater evolutionary age; they appear to be the oldest group of spirorbins (Macdonald & Rouse *in review*), and perhaps have had more time to diversify. As for the Pileolariini, these species may reap the advantages of high brood turnover (Table 4-3) and relatively size-independent fecundity (Fig. 4-2).

However, tube brooders have advantages as well, namely the ability to reproduce at much smaller body sizes (and likely earlier); this advantage may explain their multiple origins from opercular brooding ancestors (Chapters 2 & 3), but does little to explain their lower species diversity. Perhaps patterns of diversification have more to do with ecological factors such as dispersal or habitat variability. Given both the Januini and the Pileolariini are mostly tropical in distribution (Knight-Jones & Knight-Jones 1984), this suggestion seems to have merit.

4.4.4 Future studies

Many possible avenues remain for comparative studies of reproductive traits in spirorbin tubeworms. These include total lifespan, the total number of broods per lifetime, age at first reproduction (and not just minimum size), growth rates, and energetic content of eggs (this is generally not correlated with egg size; Bridges 1993). These, in combination with traits studied here, may provide a more balanced picture of the costs and benefits of different reproductive modes.

However, such a task may never be accomplished in its entirety considering the high level of intraspecific variation observed here, the causes of which remain unknown. We must also consider other constraints on reproduction, such as fertilization rates, transfer to a reproductive structure (perhaps less likely in an opercular brood chamber), and those affecting juvenile and adult survival (*e.g.*, larval settlement and predation rates). A broader sampling of species from all tribes, especially tube-brooding ones, is also needed for an accurate picture of trends in spirorbin evolution.

The costs and benefits of life history traits remain a difficult area of study, considering the number of possible evolutionary tradeoffs in optimizing reproduction; so definitive conclusions for spirorbin polychaetes remain elusive. However, we must view such comparative studies in a phylogenetic context, otherwise we may be misled by our assumptions regarding the evolution of the morphology we are studying (McHugh &

Rouse 1998; Rouse 2000). Advantages to tube brooding in spirorbins have previously been overlooked in light of our assumptions of the 'novelty' of opercular brooding.

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CHAPTER 5.

EVOLUTION OF COILING DIRECTION IN THE DIMORPHIC *PARADEXIOSPIRA VITREA* (FABRICIUS, 1780) (CIRCEINI, SPIRORBINAE)

5.1 INTRODUCTION

Examples of conspicuous bilateral asymmetry occur in all groups of organisms. Often their origin can be explained by a requirement of their environment or habits, *e.g.*, the need for eye-sidedness in flatfish (Policansky 1982), or the 'fast claw' and 'slow [crusher] claw' of lobsters (Govind 1989). However, the evolution of *directional asymmetry* (DA), or the bias towards a particular side (handedness), seems more difficult to explain: for a structure to evolve via natural selection, the existing phenotypic variation must affect reproductive success (Palmer 1996a). It is difficult to imagine why asymmetry in a particular direction (left or right) should be favored over the other (*e.g.*, right-handedness in humans and other vertebrates; Dill 1977). Even artificial selection cannot produce deviations from bilateral asymmetry in a particular direction (Palmer 1996b; based on experiments with various morphological characteristics of *Drosophila* (Coyne 1987, Maynard Smith & Sondi 1960, Purnell & Thompson 1973, Tuinstra *et al.* 1990). However, in many species, the direction of asymmetry is genetically fixed (*e.g.*, shell coiling in gastropods; Shizabaki *et al.* 2004). So how did DA evolve?

Palmer (2004) suggests that DA has arisen just as many times through genetic assimilation (Waddington 1953; 'phenotype-precedes-genotype') as it has through conventional pathways (genotype-preceding-phenotype). He suggests that the bias towards *a particular side* could be genetically fixed through an intermediate phenotype of antisymmetry (AS), the random distribution of left- and right-handed individuals within a species (characterized by a 50:50 distribution of each). Direction of asymmetry in antisymmetric species is usually induced by an environmental trigger (*i.e.*, a result of developmental plasticity), and almost never inherited (Palmer 2004). If this hypothesis is correct, we would expect antisymmetry to be ancestral (and direction of asymmetry not heritable) and directional asymmetry to be derived (and direction genetically fixed). Palmer (1996a) found support for the occurrence of this pathway to DA in various groups of organisms, including spirorbin polychaetes (supported in sections 2.3.3 and 2.4.7).

The appeal of studying bilateral asymmetries is clear: they are comparable across diverse phyla (Palmer 2004) so this allows us to make broad inferences about evolution. However, we still do not understand the nature of asymmetry in many organisms. For example, is direction of asymmetry in directionally asymmetry speices always genetically fixed, and is direction of asymmetry in antisymmetric species never heritable?

Spirorbin tubeworms present an opportunity to address the question of inheritance of directional bias. Their distinctive coiled tubes and the associated conspicuous body asymmetry make them unique among annelids. Most extant species are directionally asymmetric, with fixed (or predominantly) dextral or sinistral coiling (see Fig. 4-1 for examples of dextral and sinistral tubes). However, some species are not directionally asymmetric, but instead are dimorphic, coiling both dextrally and sinistrally.

Neomicrorbis azoricus Zibrowius 1972 is often considered the only truly antisymmetric spirorbin polychaete, as other dimorphic species have unequal proportions of dextral and sinistral individuals (biased antisymmetry in the sense of Palmer 2005). The less common coiling direction is usually reported as *situs inversus*, or a rare reversal in coiling. In these species nothing is known about whether reversed coiling direction is inherited, or if this is a result of developmental plasticity. The existence of dimorphic (dextral and sinistral) populations of *Paradexiospira vitrea* (Fabricius, 1780) in the Northeast Pacific presents an opportunity to investigate this question.

Knight-Jones *et al.* (1979) considered the dextral and sinistral forms of *Paradexiospira vitrea* (tribe Circeini) to be morphologically identical, with the exception of coil direction. Their tube morphology, operculum, and general body morphology are identical (Fig. 5-1); as is their chaetation (pers. obs.). Although *P. vitrea* is 'mostly dextral' throughout its range, the proportion of dextral and sinistral individuals has not been quantified at the population level, and the genetic basis of coiling direction remains uninvestigated.

In this study, two alternative hypotheses were addressed: (1) *P. vitrea* is antisymmetric, and therefore coiling direction is a result of random variation, or (2) coiling direction has a genetic basis. Multiple approaches were used to discern these possibilities. Breeding studies tested whether coiling direction had a genetic basis. Abundance and distribution patterns tested whether *P. vitrea* coiling forms were randomly distributed (*i.e.*, antisymmetric) and also tested for evidence of ecological segregation of the two coiling morphs. Reconstruction of the phylogenetic relationships of dextral and sinistral forms tested for evidence of divergence below the 'species level'.

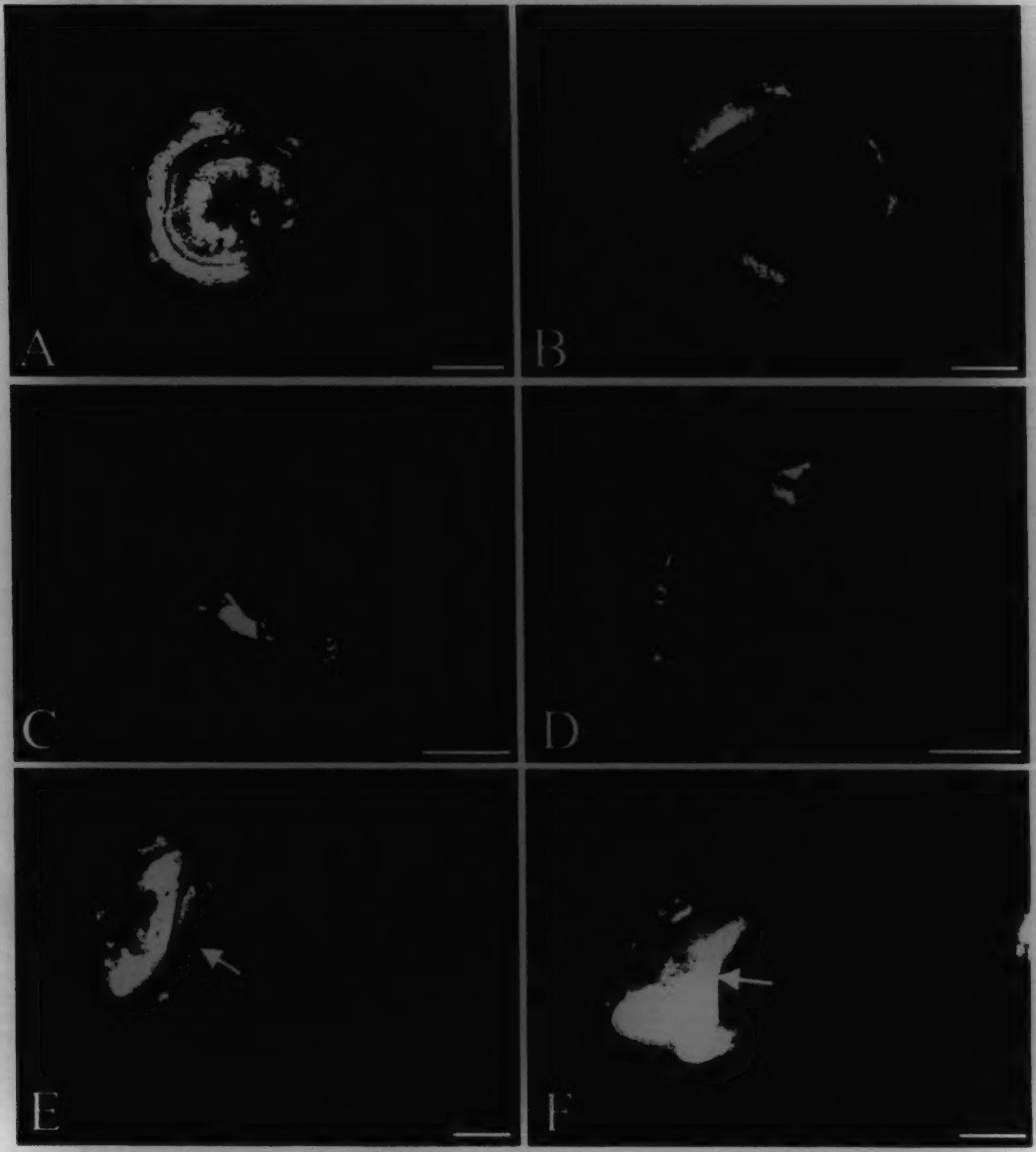

Fig. 5-1 Morphology of dextral and sinistral forms of *Paradexiospira vitrea* from Barkley Sound, BC. **A.** sinistral tube, **B.** dextral tube, **C**. whole sinistral specimen, **D.** whole dextral specimen. **E.** operculum of sinistral form, **F.** operculum of dextral form. Arrows indicate the distinctive shallow opercular plate lacking a talon. Scale bars 1mm except E and F, 100 μm.

5.2 METHODS AND MATERIALS

5.2.1 Comparison of abundance and distribution patterns

To determine the frequency of dextral and sinistral individuals in the field, I collected rocks from cobble beaches along a 20 m transect laid parallel to the shore at three shore heights within the *Paradexiospira vitrea* zone: low (0.3 ± 0.3 m), mid (0.6 ± 0.3 m) and high (0.9 ± 0.3 m), during July 2004 and 2005 (post-reproductive season) at 5 sites located in Barkley Sound, British Columbia (Fig. 5-2) (2004: Bamfield Inlet, Grappler Inlet, and Dixon Island and 2005: Bamfield Inlet, Dixon Island, and Aguilar Point).

At each metre point (20 per transect, 60 per site), the closest removable rock or shell with *P. vitrea* on it was collected. The total dextral and sinistral *P. vitrea* were counted, and the habitable surface area for *P. vitrea* was calculated for the collected substrates. Thus the relative frequency of sinistral individuals, their density, and distribution could be determined for each population. Habitable area (cm^2) (for calculation of density) was estimated roughly by measuring all sides of the rocks (or shell) with *P. vitrea* attached (assuming that sides without any settlers were not available as substrate for settling larvae).

The effects of year, site and shore height on the proportion of sinistral *P. vitrea* were analyzed using a one-way ANOVA in $\log_{10}$ transform of the data (transformed to meet assumptions of normality and homogeneity of variances).

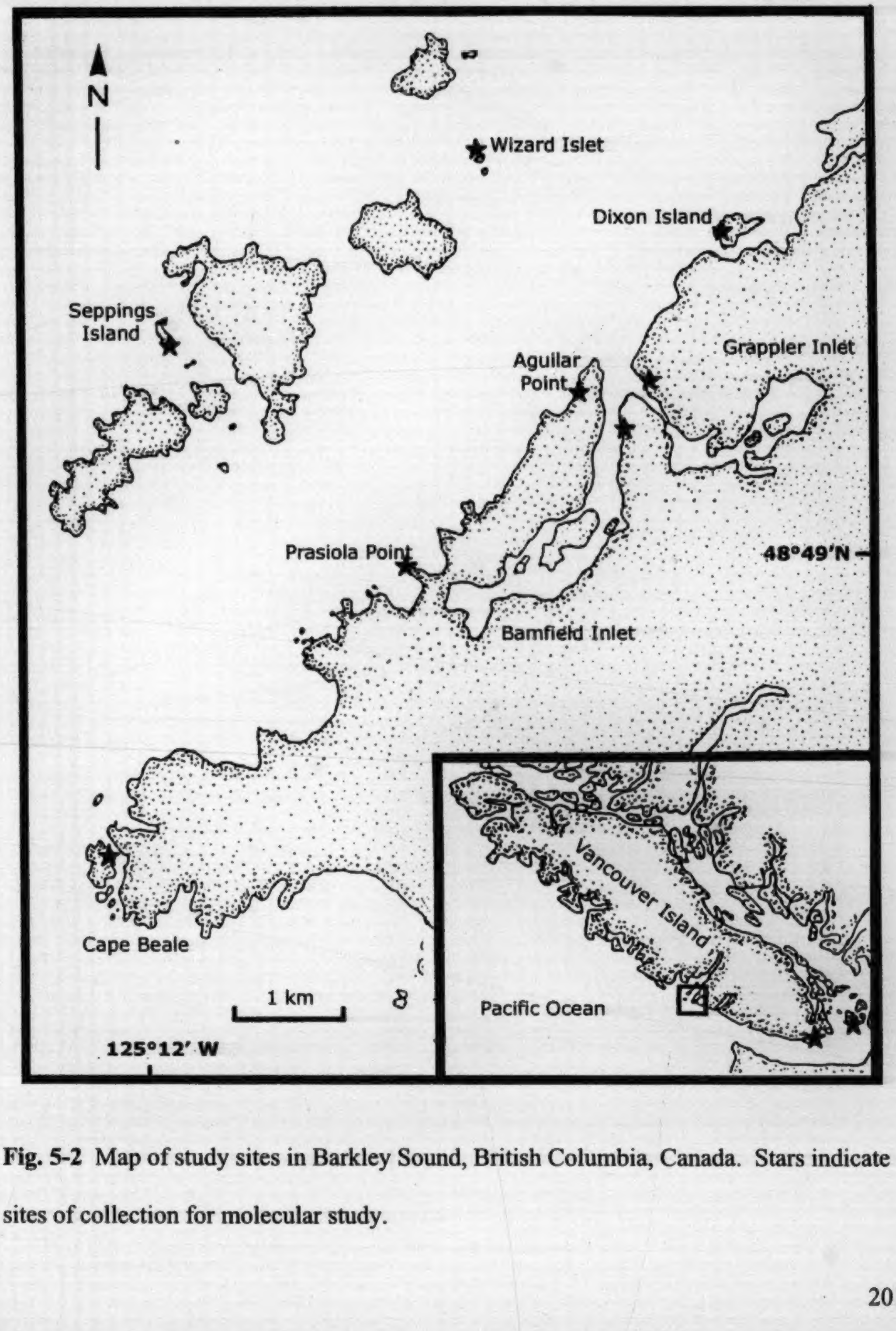
N
Wizard Islet
Dixon Island
Seppings Island
Grappler Inlet
Aguilar Point
Prasiola Point
48°49'N
Bamfield Inlet
Cape Beale
1 km
125°12' W
Vancouver Island
Pacific Ocean

Fig. 5-2 Map of study sites in Barkley Sound, British Columbia, Canada. Stars indicate sites of collection for molecular study.

5.2.2 Molecular phylogenetic relationships of dextral and sinistral morphs

To investigate the possibility that dextral and sinistral morphs may form distinct genetic lineages, cytochrome B oxidase (Cyt B; a mitochondrial gene) sequences were extracted and analyzed.

Taxon sampling - The data set consisted of 41 sampled specimens; 35 ingroup *Paradexiospira vitrea* (Barkley Sound, Victoria Harbour and San Juan Island and Finnøy, Iceland), and 6 specimens in 4 outgroup taxa (Table 5-1). Dextral and sinistral representatives of the ingroup *P. vitrea* were sequenced for each site with the exception of Wizard Islet (Barkley Sound), and Finnøy, where only dextral individuals were found.

Outgroup taxa include other representatives of the Circeini, as well as members of the Spiorbini and Pileolariini (that together are hypothesized to be sister to the Circeini; (Macdonald & Rouse *in review*)). Unfortunately, no other *Paradexiospira* species were available for use as outgroups. The addition of more outgroups (of different spirorbin tribes) did not affect ingroup topology but made the alignment more ambiguous.

Collection and preservation – Specimens of *P. vitrea* collected did not encompass the entire geographic range of the species (Knight-Jones & Fordy 1979), but instead focused on populations from Southern Vancouver Island (Table 5-1; Fig. 5-2). Specimens were collected in British Columbia (Barkley Sound and Victoria Harbour), Washington (Friday Harbor), and Iceland (Finnøy) between 2001 and 2005. Specimens were preserved in 95% ethanol for DNA extraction. Individuals from each site were also preserved in formalin as voucher specimens. Samples remained frozen at –20°C until this study commenced.

Table 5-1 *Paradexiospira vitrea* used in molecular study of relationships among dextral and sinistral morphs.

Taxon	Site	Accession Number
Outgroups		
Simplaria potswaldi (Pileolariini)	Dixon Island, Barkley Sound, BC	DQ875879
Spirorbis bifurcatus (Spirorbini)	Bamfield Inlet, Barkley Sound, BC	DQ875880
Spirorbis bifurcatus (Spirorbini)	Cattle Point, San Juan Island, WA	DQ875881
Circeis spirillum (Circeini)	Stykkishlómør, Iceland (coll. G. Rouse)	DQ875882
Circeis armoricana (Circeini)	Dixon Island, Barkley Sound, BC	DQ875883
Circeis armoricana (Circeini)	Cattle Point, San Juan Island, WA	DQ875884
Ingroup *Paradexiospira vitrea*		
Dextral		
FI-D-1	Finnøy, Iceland (coll. G. Rouse)	DQ875887
FI-D-2	Finnøy, Iceland (coll. G. Rouse)	DQ875888
BI-D-1	Bamfield Inlet, Barkley Sound, BC	DQ875889
BI-D-2	Bamfield Inlet, Barkley Sound, BC	DQ875890
BI-D-3	Bamfield Inlet, Barkley Sound, BC	DQ875891
BI-D-4	Bamfield Inlet, Barkley Sound, BC	DQ875892
BI-D-5	Bamfield Inlet, Barkley Sound, BC	DQ875893
GI-D-1	Grappler Inlet, Barkley Sound, BC	DQ875894
GI-D-2	Grappler Inlet, Barkley Sound, BC	DQ875895
GI-D-3	Grappler Inlet, Barkley Sound, BC	DQ875896
DI-D-1	Dixon Island, Barkley Sound, BC	DQ875902
DI-D-2	Dixon Island, Barkley Sound, BC	DQ875903
AP-D-1	Aguilar Point, Barkley Sound, BC	DQ875904
AP-D-2	Aguilar Point, Barkley Sound, BC	DQ875905
CP-D-1	Cattle Point, San Juan Island, WA	DQ875897
CP-D-2	Cattle Point, San Juan Island, WA	DQ875898
VH-D	OgdenPoint, Victoria Harbor, BC	DQ875899
CB-D	Cape Beale, Barkley Sound, BC	DQ875900
PP-D	Prasiola Point, Barkley Sound, BC	DQ875901
WI-D-1	Wizard Islet, Barkley Sound, BC	DQ875885
WI-D-2	Wizard Islet, Barkley Sound, BC	DQ875886
Sinistral		
BI-S-1	Bamfield Inlet, Barkley Sound, BC	DQ875914
BI-S-2	Bamfield Inlet, Barkley Sound, BC	DQ875915
GI-S-1	Grappler Inlet, Barkley Sound, BC	DQ875912
GI-S-2	Grappler Inlet, Barkley Sound, BC	DQ875913
CP-S-1	Cattle Point, San Juan Island, WA	DQ875916
CP-S-2	Cattle Point, San Juan Island, WA	DQ875917
CP-S-3	Cattle Point, San Juan Island, WA	DQ875918
CP-S-4	Cattle Point, San Juan Island, WA	DQ875919
CB-S-1	Cape Beale, Barkley Sound, BC	DQ875909
CB-S-2	Cape Beale, Barkley Sound, BC	DQ875910
CB-S-3	Cape Beale, Barkley Sound, BC	DQ875911
PP-S-1	Prasiola Point, Barkley Sound, BC	DQ875906
PP-S-2	Prasiola Point, Barkley Sound, BC	DQ875907
PP-S-3	Prasiola Point, Barkley Sound, BC	DQ875908

DNA extraction, amplification and sequencing - Entire worms were used for DNA extraction. The majority of epibionts and visible traces of digestive tract were removed under a light microscope using a sterile scalpel and forceps. The remaining tissue was used for extraction. Voucher specimens were from the same site and were preserved in both ethanol and formalin for possible future study. To remove traces of ethanol, the tissue was rinsed in 1X Phosphate Buffered Saline (PBS) three times, and left to soak for approximately 1 hour at the last rinsing step. Genomic DNA was extracted using a Qiagen DNA Mini Kit ™ (Qiagen Inc.) and eluted in 30-50 µL of sterile distilled water.

Cytochrome b, a mitochondrial gene, was amplified directly from these extractions in a single 400 bp fragment. PCR reactions were 25 µL and contained the following: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2-4 mM MgCl2, 0.5 µM each primer (Table 5-2), 100 µM each dNTP, 1.5 Units Taq (M. Pickard, University of Alberta) and 20-100 ng template DNA (usually 1-2 µL). The following PCR temperature profile was used: 95°C for 2 min, 35 cycles of 94°C for 30 sec, 47-49°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 7 min.

Amplification products were separated electrophoretically on a 1.1% agarose gels in TAE buffer, and stained with ethidium bromide. PCR products were either purified directly with a PCR Purification Kit (Qiagen Inc.) or bands were excised from the gel and purified with a QIAQuick™ Gel Extraction Kit (Qiagen Inc.). Elution was in sterile distilled water in both cases.

Sequences were obtained directly with the BigDye v 3.1 Cycle Sequencing Kit (Applied Biosystems). Full reactions were 20 μL: 2μL Big Dye, 6 μL buffer (200 mM Tris-HCl (pH 9.0), 5 mM $MgCl_2$), 5 μL 1μM primer (Table 5-2), and 1-6 μL PCR product. Cycling sequencing was done according to the manufacturer's instructions, and separated on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Basecaller v.3.4.1 was used to read the chromatograms and GeneTool 2.0 to assemble gene fragments.

Alignment - Sequences were aligned using Clustal W (Thompson *et al*., 1994) with gap opening and extension penalties of 8 and 5 respectively (pairwise and multiple). These values minimized the number of ambiguously aligned bases. Alignments were edited by eye in MacClade 4.07 (Maddison & Maddison, 2005), with no sites excluded in the final analysis. The alignment of 591 bp is available from TreeBase (www.treebase.org).

Phylogenetic analysis - Maximum Parsimony (MP) and Likelihood (ML) analyses were conducted in PAUP*4.0b10 (Swofford 2003). Parsimony analyses were unweighted, and performed with heuristic searches (random stepwise addition, TBR branch-swapping, multrees in effect, with 10 trees held at each step). Bootstrap support was found with 1000 replicate searches.

Table 5-2 Primers used in the amplification and sequencing of *Paradexiospira vitrea* cytochrome B oxidase. Position refers to the alignment used in this study.

Primer	Position	Sequence (5'-3')	Reference
Forward			
CB-FA	1-27	GGW TAY GTW YTW CCW TGR GGW CAR AT	Boore & Brown 2000
CB-FB	1-27	GGT TAT GTT TTT CCA TGG GGW CAG AT	This study
CB-FC	8-31	YWY TRC CTT GRG GRC ARA TAT C	T. Dahlgren, pers. comm.
Reverse			
CB-RA	549-572	GCR WAY ARA AAR TAY CAY TCW GG	T. Dahlgren, pers.comm..
CB-RB	549-578	GCR TAW GCR AAW ARR AAR TAY CAY TCW GG	Boore & Brown 2000
CB-RC	549-578	GCR TAA GCG AAA AGR AAG TAC CAC TCA GG	K. Halanych pers. comm.

Maximum Likelihood analyses were run with the model HKY + I + Γ as selected by the Akaike Information Criterion (AIC) in Modeltest 3.06 (Posada & Crandall, 1998). This model and the given parameter estimates (Table 5-3) for each data set were used to obtain an ML topology in PAUP* (Heuristic searches, TBR, random addition, 1 tree held at each step).

Bayesian analysis were run under the HKY + I + Γ model, with Mr. Bayes 3.0b4 (& Ronquist 2001). Four Markov Monte Carlo chains (three heated and one cold) were run simultaneously for 1,100,000 generations with sample frequency of 1000. One hundred trees were discarded as *burnin*, thus the final topology, support values, and parameter values were calculated from a total of 1000 trees.

Decay support (Bremer 1994) was determined using the program Autodecay 4.0.2 (Ericksson 1998).

5.2.3 Inheritance of coiling direction

To investigate whether coiling direction variation was heritable, the coiling direction of offspring from dextral and sinistral parents was monitored throughout May and June 2005. Single *Paradexiospira vitrea* were removed from their substrate with a scalpel and placed in tissue culture dishes of filtered seawater (only undamaged worms were used; a total of 50 for each coiling direction). These were then placed in shallow seawater tables and their water changed daily for a period of one month. They were monitored every second day for larval settlement. After one week, non-reproductive individuals were replaced to ensure that a total of 50 broods were scored.

Table 5-3 Parameter estimates for evolution of Cytochrome b oxidase sequences based on HKY + I + Γ model

Parameter	ML estimate	Bayesian Estimate (95% CI)
- ln *L*	7739.0542	7751.5680 (7744.1822, 7758.8982)
Ti/Tv ratio	1.1929	2.4450 (2.3082, 2.5848)
π_A	0.2266	0.2260 (0.2172, 0.2348)
π_C	0.2278	0.2283 (0.2195, 0.2371)
π_G	0.2371	0.2381 (0.2291, 0.2472)
π_T	0.3085	0.3076 (0.2974, 0.3178)
gamma	1.9794	2.1302 (1.7943, 2.4662)
pinvar	0.0585	0.0582 (0.0395, 0.0769)

To increase the possibility of observing a rare coiling morphology (*i.e.*, sinistral offspring of a dextral parent or *vice versa*), large monocultures of sinistral and dextral worms (~100 individuals) were also monitored. These cultures were created by scraping all individuals of one coiling direction off small rocks (three cultures of each coiling direction) and were maintained in an incubator (14°C, 15 h light/9 h dark) in filtered seawater for a period of 8 months (fed weekly with a suspension of nutritional yeast). Three mixed cultures were also monitored (each containing approximately 50 individuals of each coiling direction). Cultures were monitored weekly and later bi-weekly (the rocks examined under a microscope) to detect any infidelity to parental coiling direction.

5.2.4 Reproductive timing of sinistral and dextral morphs

The reproductive timing of dextral and sinistral populations may give evidence of reproductive isolation, and thus evidence of a possible speciation event between the two coiling morphs. Forty dextral and sinistral individuals were collected bi-weekly from the mouth of Bamfield Inlet and dissected to determine the proportion of reproductive individuals.

5.3 RESULTS

5.3.1 Abundance and distribution patterns

The sinistral form of *P. vitrea* was less common than the dextral form at all sites. Dextral worms were found at a mean density 0.49±0.03 worms/cm^2 (N= 5 sites), and the sinistral form was found at a mean density of 0.039±0.005 worms/cm^2 (Table 5-4). Sinistral individuals consistently made up less than 20% of the *P. vitrea* population (11.1±0.01% of the population across all sites). In addition, at sites where *P. vitrea* distribution was not quantified, the sinistral form was much less common, including Ohiat Island, Barkley Sound; Cattle Point, Friday Harbor, San Juan Island; as well as various sites in the Queen Charlotte Islands (pers. obs.). The densities of dextral and sinistral forms were correlated across sites (Pearson correlation coefficient= 0.142, $p< 0.01$). Thus the distribution of dextral and sinistral *P. vitrea* was not consistent with the expected 50:50 distribution of left and right individuals characteristic of antisymmetry (Table 5-3).

Significantly, the sinistral forms all occurred in higher proportion with increasing shore height within the *P. vitrea* zone (Figs. 5-3 and 5-4). This pattern persisted at all sites, and in both years. The effect of year on the proportion of sinistral individuals was not significant (ANOVA, $p= 0.50$), and was removed from the analysis. After the removal of year, both the effect of site ($p< 0.01$) and shore height were highly significant (two-way ANOVA; $p< 0.01$ in both cases), as was their interaction ($p< 0.001$). Multiple comparisons (Tukey's HSD) revealed that Aguilar Point had significantly different proportions of sinistral *P. vitrea* than Bamfield and Grappler Inlets, and Dixon Island.

Table 5-4 Density of dextral and sinistral *Paradexiospira vitrea* in Barkley Sound, BC

Site	Year	Density (number of worms/cm^2)	
		Dextral	Sinistral
Dixon Island	2004	0.657 ± 0.369	0.021 ± 0.065
Dixon Island	2005	0.416 ± 0.635	0.009 ± 0.015
Bamfield Inlet	2004	0.395 ± 0.355	0.031 ± 0.065
Grappler Inlet	2004	0.694 ± 0.954	0.112 ± 0.192
Aguilar Point	2005	0.388 ± 0.638	0.027 ± 0.055
Seppings Island	2005	0.388 ± 0.404	0.033 ± 0.062

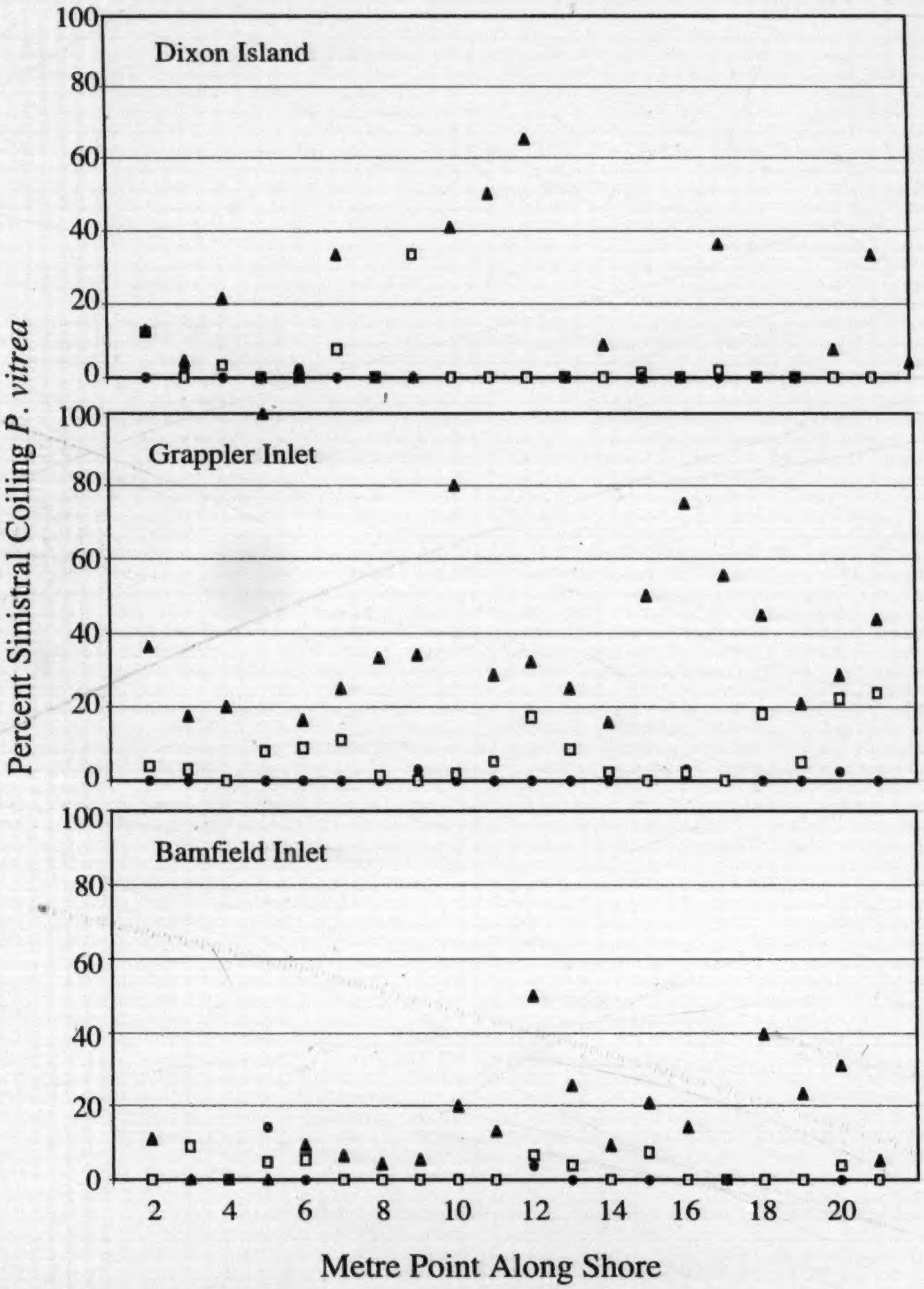

Fig. 5-3 Proportion of total *Paradexiospira vitrea* that were sinistral at three shore heights: high (black triangles; ~ 0.9 m), mid (open squares; ~ 0.6 m) and low (gray circles; ~0.3 m). Transects (20 m) were laid parallel to the shore across the *P. vitrea* zone at three sites in Barkley Sound, B.C. during 2004.

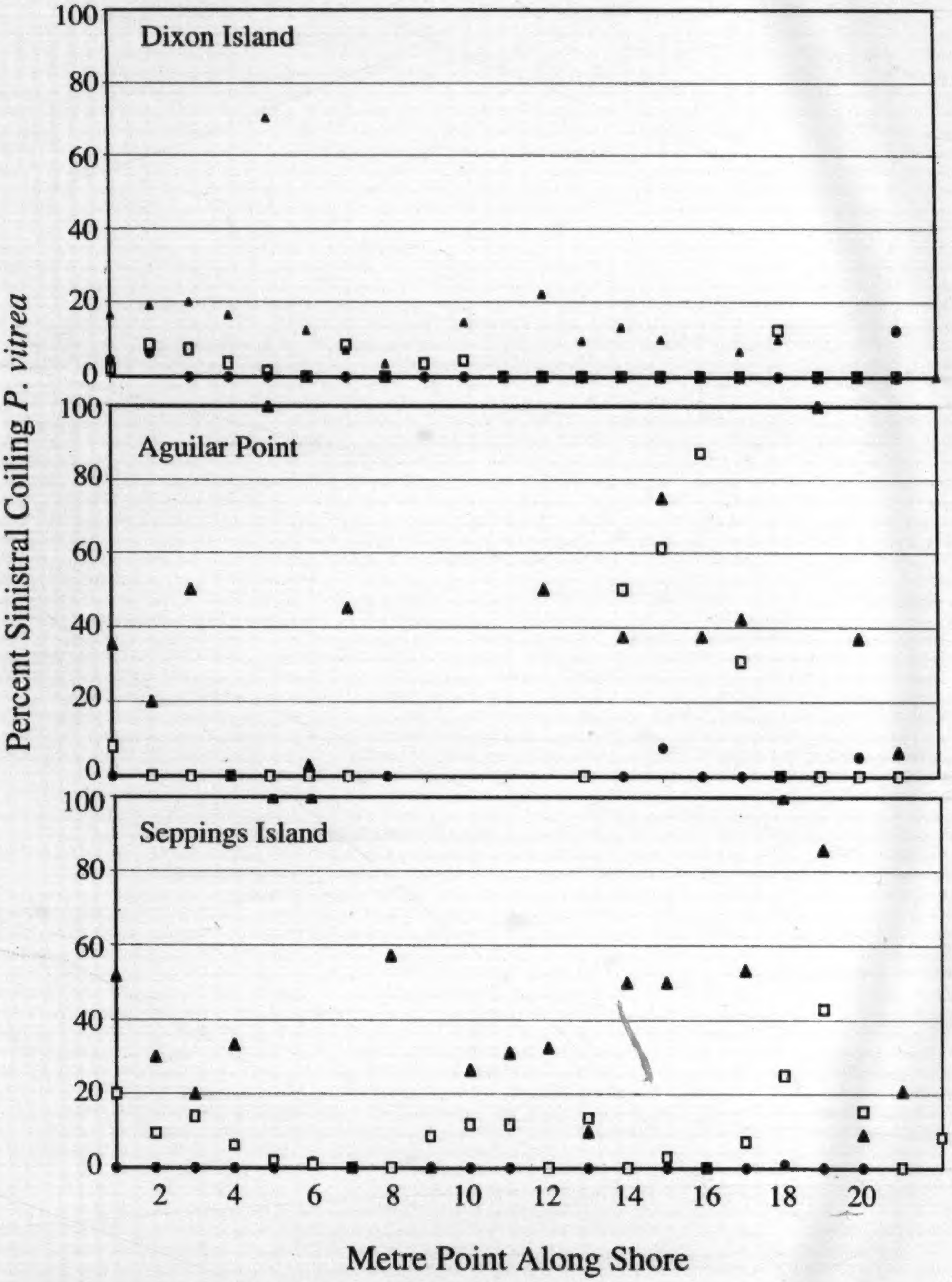

Fig. 5-4 Proportion of total *Paradexiospira vitrea* that were sinistral at three shore heights: High (black triangles; ~ 0.9 m), Mid (open squares; ~ 0.6 m) and Low (gray circles; ~0.3 m). Transects (20 m) were laid parallel to the shore across the *P. vitrea* zone at three sites in Barkley Sound, B.C. during 2005.

Seppings Island was also significantly different from Dixon ($p < 0.05$ in all cases). The frequency of sinistral *P. vitrea* was significantly different among the three shore heights (low, mid, and high; Tukey's HSD, $p < 0.05$ in all cases). Thus although the percentage of sinistral *P. vitrea* did not necessarily differ among sites, the percentage of sinistrals were always significantly higher at the upper shore level, so the dextral and sinistral forms appear to be ecologically segregated.

5.3.2 Phylogenetic relationships of dextral and sinistral Paradexiospira vitrea

All analyses, including MP, ML and Bayesian inference, inferred *P. vitrea* to be monophyletic with respect to the outgroup taxa (Fig. 5-5). This grouping had moderate support (Bayesian posterior probability (pp)= 0.82, MP Bootstrap (MPBS)= 67, Decay index (DI)= 2). Within this group, the sinistral form was a distinct monophyletic clade, with strong statistical support (pp= 1.0, MPBS= 100, DI= 23). Significantly, the sinistral *P. vitrea* formed a distinct clade within the dextral *P. vitrea*.

The sister group to the sinistral clade were the two dextral *P. vitrea* from Bamfield Inlet (of 5 total; BI-D-4 and –5), Wizard Islet (WI-D-1 and –2) and Finnøy, Iceland (FI-D-1 and -2) (sister relationship with pp= 0.99, MPBS= 65, DI= 7). The dextral individuals collected in Bamfield Inlet were therefore not monophyletic, as the other three individuals were found nested within the clade sister to the clade of sinistral specimens (BI + FI + WI + Sinistral) (pp= 0.96, MPBS= 68, DI= 4).

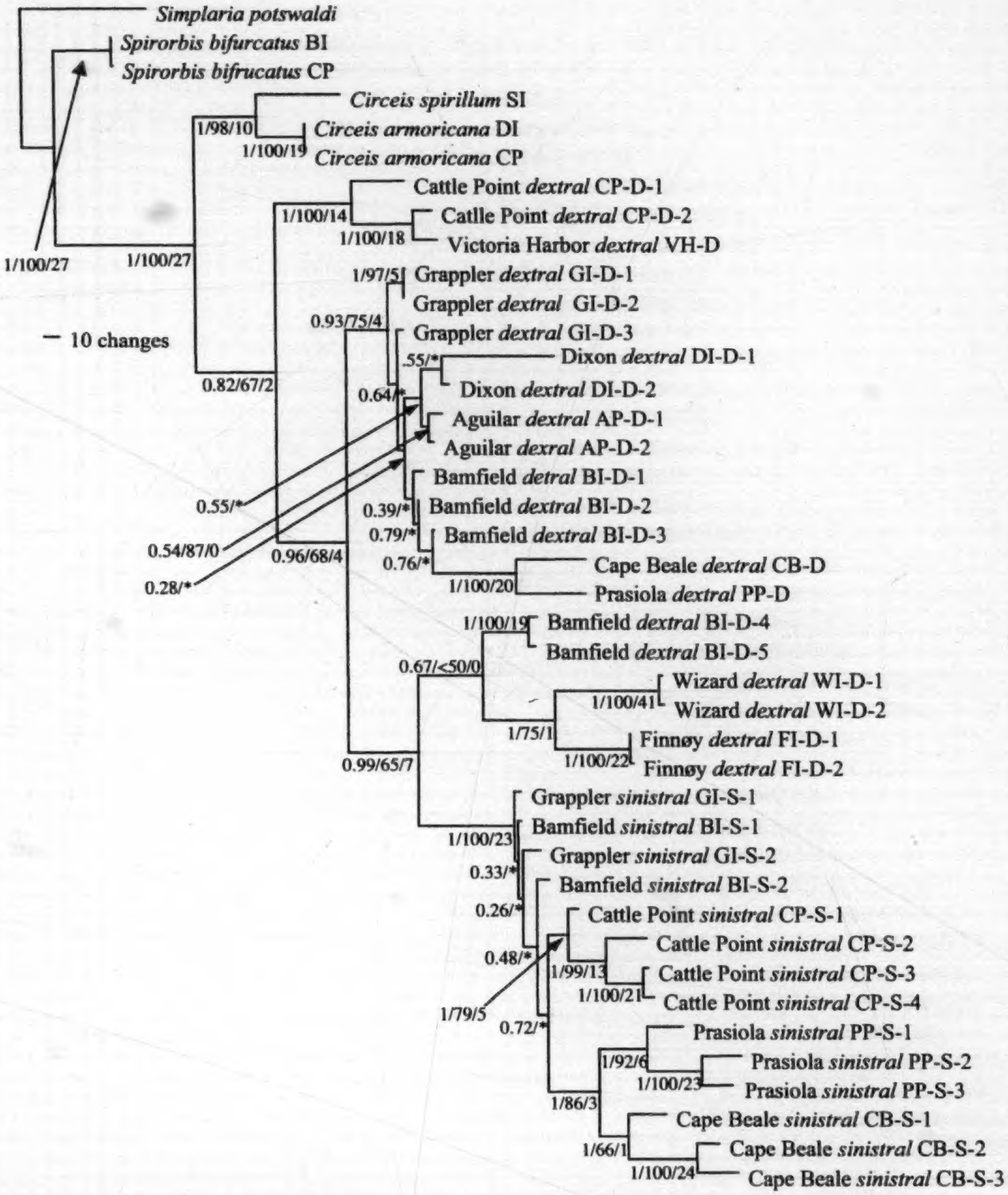
Simplaria potswaldi
Spirorbis bifurcatus BI
Spirorbis bifrucatus CP
Circeis spirillum SI
Circeis armoricana DI
Circeis armoricana CP
Cattle Point dextral CP-D-1
Catlle Point dextral CP-D-2
Victoria Harbor dextral VH-D
Grappler dextral GI-D-1
Grappler dextral GI-D-2
Grappler dextral GI-D-3
Dixon dextral DI-D-1
Dixon dextral DI-D-2
Aguilar dextral AP-D-1
Aguilar dexral AP-D-2
Bamfield detral BI-D-1
Bamfield dextral BI-D-2
Bamfield dextral BI-D-3
Cape Beale dextral CB-D
Prasiola dextral PP-D
Bamfield dextral BI-D-4
Bamfield dextral BI-D-5
Wizard dextral WI-D-1
Wizard dextral WI-D-2
Finnøy dextral FI-D-1
Finnøy dextral FI-D-2
Grappler sinistral GI-S-1
Bamfield sinistral BI-S-1
Grappler sinistral GI-S-2
Bamfield sinistral BI-S-2
Cattle Point sinistral CP-S-1
Cattle Point sinistral CP-S-2
Cattle Point sinistral CP-S-3
Cattle Point sinistral CP-S-4
Prasiola sinistral PP-S-1
Prasiola sinistral PP-S-2
Prasiola sinistral PP-S-3
Cape Beale sinistral CB-S-1
Cape Beale sinistral CB-S-2
Cape Beale sinistral CB-S-3
1/98/10
1/100/19
1/100/14
1/100/18
1/100/27
1/100/27
1/97/5
0.93/75/4
— 10 changes
.55/*
0.82/67/2
0.64/*
0.55/*
0.39/*
0.79/*
0.54/87/0
0.96/68/4
0.76/*
0.28/*
1/100/20
1/100/19
0.67/<50/0
1/100/41
1/75/1
1/100/22
0.99/65/7
1/100/23
0.33/*
0.26/*
0.48/*
1/99/13
1/100/21
1/79/5
0.72/*
1/92/6
1/100/23
1/86/3
1/66/1
1/100/24

Fig. 5-5 Maximum likelihood (HKY + I + Γ) topology inferred from 591 bp of cytochrome B oxidase sequence (ML Tree Length= 7739.0542, MP TL= 1719, 144 trees, CI= 0.44). Support values above or below nodes are Bayesian posterior probability/maximum parsimony bootstrap support/Bremer decay index. Asterisks denote disagreement among methods of reconstruction. Specimens labeled –S- were sinistral, those labeled –D- were dextral. See Table 5-1 for locality information.

In all other cases, individuals of the same coiling direction from the same site grouped together either in monophyletic groups (Dextral: Grappler Inlet (GI), Dixon Island (DI), Aguilar Point (AP) and Cattle Point (CP); Sinistral: Prasiola Point (PP), Cape Beale (CB), and Cattle Point (CP) or in paraphyletic grades (Sinistral: GI and BI). In most cases, sites with closest proximity grouped together (Dextral: Victoria Harbour (VH) grouping with CP, and CB with PP (both coiling directions).

The phylogenetic relationships among dextral and sinistral individuals were not always consistent across sites. Dextral individuals from Cattle Point were ancestral to all other *P. vitrea* (pp= 0.96, MPBS= 68, DI= 4), whereas sinistral individuals from Cattle Point were nested within the sinistral clade, closely related to those from Grappler and Bamfield Inlets, and sister to those from Prasiola Point and Cape Beale (pp= 0.72). However, one intriguing geographic correlation emerged: in both the large dextral clade and the sinistral clade, specimens from protected locales (Grappler and Bamfield Inlets) were basal to those from more exposed shores (Capel Beale and Prasiola Point).

Some more recent splits lacked resolution, indicated by low pp values and conflict among Bayesian, ML and MP topologies. However, this does not affect conclusions about the distinctiveness of the sinistral form within dextral *P. vitrea*. Nontheless, the lack of resolution indicates a need for more sequence data to resolve these discrepancies.

5.3.3 Inheritance of coiling direction

All *P. vitrea* broods monitored (n= 50 each dextral and sinistral worms; 100 total) had 100% fidelity to the parental coiling direction. No examples of *situs inversus* were observed in the large cultures (N> 1000 juveniles). Thus coiling direction is likely genetic, and the dextral and sinistral forms appear not to interbreed. Brood sizes were similar (and highly variable) among sinistral and dextral cultures, with an average of 11.5 ± 9.3 (mean±SD; range of 3 to 35; N= 36) for the dextral cultures, and 10.5 ± 8.4 for sinistral cultures (range of 2 to 32; N= 36). These brood sizes are less than half those observed in Chapter 4 (Table 4-1); likely because broods were reared in the lab during low reproductive season.

5.3.4 Reproductive season

Dextral and sinistral *P. vitrea* had overlapping reproductive seasons in Bamfield Inlet (Fig. 5-6), but, surprisingly, the dextral form was reproductive all year. It remained reproductive, albeit at a low level (~20-30% of individuals reproductive), throughout the summer and autumn months (July-November), whereas the sinistral form was not reproductive at all during this time. Both had their peak of reproduction in the spring (April and May). The dextral morph may not have as high a reproductive peak as the sinistral form (100% in late April); but this is not clear from these data.

It also appears the peak reproductive season of the dextral morph may be slightly later than that of the sinistral morph; the sinistral morph started increasing its level of reproduction in early November, whereas the dextral morph started increasing its level of reproduction in early December.

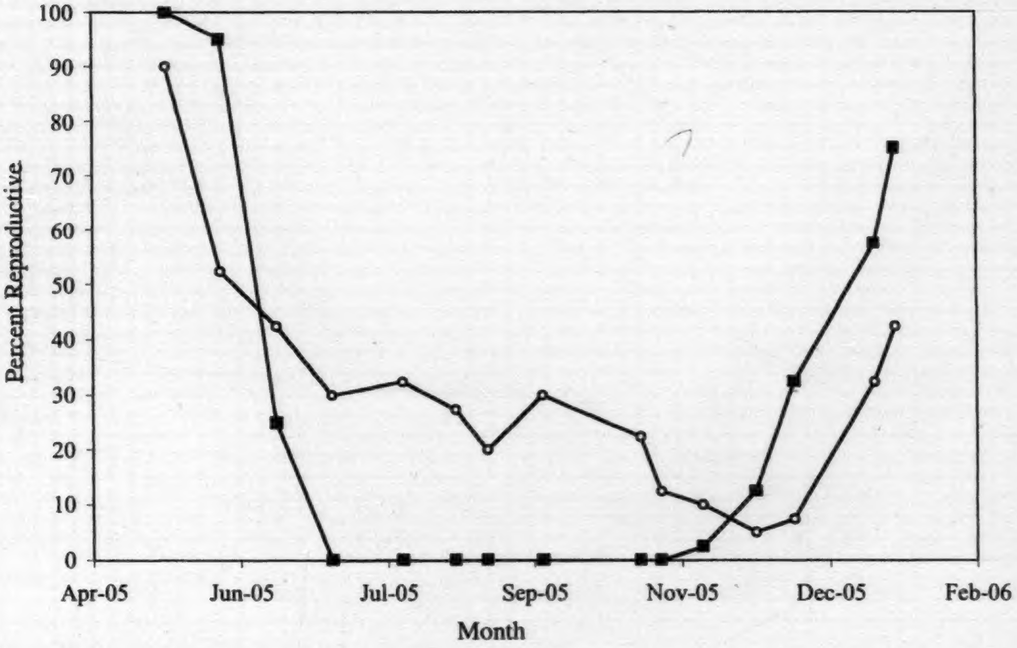

Fig. 5-6 Percent of of dextral (black sqares) and sinistral (open circles) individuals of *Paradexiospira vitrea* found to be brooding in Bamfield Inlet, Barkley Sound, British Columbia.

5.4 DISCUSSION

5.4.1 A not-so-cryptic species?

Although the assumption that sinistral and dextral morphs of *Paradexiospira vitrea* are the same species seems reasonable given their morphological similarity (*e.g.*, Knight-Jones *et al.* 1979; Fig. 5-1), their coiling direction is an indication of deeper divergence. All evidence indicates the two morphs are genetically independent and ecologically isolated lineages. Thus the lower incidence of the sinistral morph (~20% of the population) is not explained by a non-random removal of one morph from the population (*e.g.,* post-settlement mortality) but more likely is a causal factor related to their distinct genetic makeup.

The sinistral morph of *P. vitrea* formed a distinct monophyletic group within a larger dextral clade based on phylogenetic reconstruction with ~500 bp of cytochrome B oxidase). Despite limited taxon sampling, this relationship had consistently high support. The genetic uniqueness of the sinistral morph is further supported by the 100% inheritance of coiling direction, based on careful observation of coiling direction of offspring of single individuals as well as large cultures (>300 individuals total).

The difference in distribution of the sinistral and dextral *P. vitrea* is remarkable: sinistral worms were consistently found in higher proportions with increasing shore height. Even at sites where the differences in *P. vitrea* populations were not quantified, only the sinistral morph was observed in high tide pools (*e.g.*, Ohiaht Island, Barkley Sound, Cattle Point, Friday Harbor; pers. obs.), where the dextral morph remained lower on the shore. These findings may indicate a higher tolerance of the sinistral form to abiotic stresses associated with increasing shore height (*e.g.*, temperature and/or desiccation), or perhaps less likely resulting from a biotic interaction, such as competitive exclusion of the sinistral form lower on the shore, or interactions with a directionally asymmetric predator. This question remains open. In the course of pursuing an answer, perhaps it would become clear if sinistrality itself limits the distribution of the sinistral morph, or if differences in direction of coil are merely coincidental.

It is also difficult to tease apart whether differences in reproductive season are a result of different distributions or different genetic makeups of each morph. The dextral morph remains reproductive year round, whereas the sinistral form does not (Fig. 5-6). Whether the sinistral form is limited by harsh abiotic stresses in the summer that inhibit brooding in the high intertidal, or whether this is reflects genetic difference among the two morphs, remains unknown. They could be responding to similar environmental cues, but their differing distributions may affect their response. More data from other sites is needed to confirm this observation.

Nonetheless, the reproductive isolation between the two morphs is a mystery, given that these worms have overlapping distributions and reproductive period. Requirements for sympatric speciation to occur (with moderate gene flow) are the selection for divergence to be strong, and mate choice must be correlated with the factor promoting divergence (Felsenstein 1981, Rice 1987). However, neither of these obviously apply to *P. vitrea*, as the advantage to a particular coiling direction is not obvious, and mate choice does not likely apply to the the spirorbin mode of reproduction. In the case of *P. vitrea*, this speciation event may have resulted from the occupation of an ice-age refugium in the Northeast Pacific and subsequent genetic drift. This hypothesis has been invoked to explain speciation events in other marine animals (*e.g.*, clingfish (Hickerson & Ross 2001), sea cucumbers (Arndt & Smith 1998), gastropods (Collins *et al.* 1996, Marko 2005), and stickleback (Deagle *et al.* 1996)). Perhaps the sinistral form diverged along the British Columbia coast and subsequently reinvaded the Pacific coast of North America. However, this remains conjecture: whether the sinistral coiling arose before, after, or contributed to, this speciation is unclear. In addition, the reason sinistrality persisted in a dextral population, and how it became genetically fixed remains unknown.

5.4.2 Implications for the evolution of directional asymmetry in the Spirorbinae

The divergence of the two *Paradexiospira vitrea* morphs, with respect to their coiling direction, is intriguing. Functional advantages to one coiling direction over another are hard to imagine. In other animals, conspicuous bilateral asymmetries can, for example, affect mating behaviour (*e.g.*, phallostethid and swordtail fish (Parenti 1996 and Morris *et al.* 2006 respectively) and snails (Asami *et al.* 1998)), fighting behaviour (fiddler crabs; Crane 1967), or predation rates (*e.g.*, of gastropods by crabs (Dietl & Hendricks 2006), but these do not do not shed light on how coiling direction may affect the sedentary, filter feeding spirorbin polychaetes.

Wisely (1960) reports competition among *Spirorbis* larvae (tribe Spirorbini) - they space themselves out at the time of settlement. Since this spacing occurs even in response to inanimate objects, it is almost certainly evoked by alien species. But Knight-Jones *et al.* (1975) point out that it is scarcely conceivable that such behaviour could lead to exclusion of one species by another at the densities seen in natural habitats, (*e.g.*, 1/cm^2, Table 5.4), especially since territorial demands of larvae become less exacting when total space is limited (Wisely 1960). These arguments seem to be unproductive - and abiotic advantages to sinistrality are even more elusive.

Directional asymmetry (DA) itself may provide some benefits, regardless of the direction of bias. The incidence of *situs inversus* of internal organs appears to have declined throughout vertebrate evolution (Palmer 2004), suggesting the existence of an evolutionary trend towards increased canalization, or robustness to perturbation (Proulx & Phillips 2005). Perhaps canalization itself is advantageous because it increases developmental stability; the actual direction of bias is more a by-product of fixation. If this were the case, we would expect the majority of spirorbin species to be directionally asymmetric (they are) and the antisymmetric precusor would be evolutionarily short-lived (it is rare; we assume short-lived). We might not expect to see a dominant coiling direction among spirorbins, which is the case: Although the majority of species are sinistral, this seems to be the result of phylogenetic effects rather than actual selection for sinistrality.

Fixed sinistrality in *P. vitrea* appears to have arisen only once on the west coast of North America. The fact that we do not see evidence of environmental influence on coiling direction here (in the form of an antisymmetric precursor, or non-genetic variation), does not exclude the possibility that this may have been a source of asymmetric forms in this species or other Spirorbinae. However, it does raise concerns about reports of *situs inversus* and antisymmetry in other species: are these reports truly referring to the same, reproductively cohesive 'species' in all cases? Even the touted antisymmetric *Neomicrorbis azoricus* Zibrowius 1972 is not immune to this question, as we see here that morphology alone cannot always delineate species. This problem may be exacerbated in spirorbin polychaetes (and perhaps annelids in general), which have few quantifiable differences in morphology to begin with. Another problem arises when looking for antisymmetric species deep within the spirorbin tree: their fossil record is unreliable, as tubes are rarely distinctive enough to discern species (Knight-Jones 1978; Knight-Jones *et al.* 1991). Thus much opportunity exists for taxonomic investigations into possible 'cryptic' species within putatively dimorphic ones.

Many opportunities also exist for investigating asymmetry variation in spirorbin polychaetes in general. A recent study of gastropod early development (Shizabaki *et al.* 2004) presents an especially intriguing research direction: shell coiling in dextral and sinistral forms *of the same species* have cleavage patterns that are not mirror-image processes, but rather exhibit different cytoskeletal movements. Perhaps the early development of dimorphic spirorbin species (or sister species) may reveal equally intriguing developmental mechanisms, as well as clues as to the origin of coiling direction reversals.

5.4.3 Biogeography of Paradexiospira vitrea

Phylogenetic reconstruction of the relations among dextral and sinistral morphs revealed some intriguing biogeographic patterns. For example, the grouping of dextral *P. vitrea* from Iceland with those from Wizard Islet, Barkley Sound (pp= 1, but low bootstrap and Bremer support (75 and 1 respectively)) was unexpected, especially considering the apparent limited dispersal ability of competent larvae (*e.g.*, Knight-Jones 1951). This analysis suggests a population structure exists below the species level that is not explained by distribution alone. Interestingly, the Wizard specimens were the only subtidal collection in Barkley Sound (~20 m), and the specimens from Iceland were also collected subtidally. Thus perhaps their local habitat (*e.g.*, intertidal or subtidal) provides important clues about their evolutionary history, as subtidal forms might represent yet another cryptic species. Reports of *P. vitrea* from the Atlantic are based on material found mostly subtidally (~15-25 m; summarized in Knight-Jones & Knight-Jones 1977), and are exclusively dextral.

The worldwide distribution of dextral *P. vitrea* suggests a unique origin of the sinistral morph in the East Pacific. *Paradexiospira vitrea* described from the Atlantic are exclusively dextral, with no reports of sinistrality. These descriptions include material from Britain (Knight-Jones & Knight-Jones 1977), Newfoundland (Bush, 1904), Russia (Uschakov 1955), Norway (Bergan 1953), Sweden (Borg 1917), and Brittany (Quiévreux 1962). Sinistral forms are reported exclusively from the Pacific coast of North America, extending from Alaska to Monterey, California (Knight-Jones *et al.* 1979).

Other spirorbin species have equally fascinating geographic patterns of asymmetry variation. For example, members of the genus *Spirorbis* (tribe Spirorbini) is typically sinistral, but a few dextral representatives are found on the West Coast of North America. These dextral species are concentrated between Mexico and Peru (Knight-Jones *et al.* 1991). Investigating these geographic patterns, as well as phylogenetic ones, can test hypotheses of speciation and dispersal.

One interesting geographic pattern that emerged is the micro-phylogeographic relations within dextral and sinistral forms from exposed and sheltered habitats. In both the major dextral clade, and the sinistral clade, specimens from sheltered habitats (*e.g.*, Bamfield and Grappler Inlets, Dixon Island, Aguilar Point and Cattle Point) were more basal to those from exposed habitats (*e.g.*, Cape Beale and Prasiola Point). These divergence patterns might possibly be adaptive responses, given that cytochrome B is a metabolic gene. Alternatively, selective pressure to maintain genes in exposed sites maybe be weaker given that more unpredictable environments may result in more metabolic states, and the number of metabolic states is associated with greater sequence divergence (Bilu 2006).

5.4.4 Morphological and molecular taxonomy

Molecular systematics is invaluable in the study of spirorbin taxonomy. Many examples exist of ecologically isolated populations that are scarcely distinguishable morphologically from sister species (*e.g.*, Knight-Jones *et al.* 1975, de Silva & Knight-Jones 1962, Knight-Jones & Knight-Jones 1977). Furthermore, we know very little about the extent of phenotypic plasticity of traditional taxonomic characters (*e.g.*, tube and operculum; Knight-Jones 1978). Thus differentiating among spirorbin species has posed a problem to many taxonomists, and this has resulted in a confusing and convoluted taxonomic history.

The history of spirorbin taxonomy offers a useful lesson in the labile nature of our understanding of morphological characters, particularly coiling direction. In early descriptions of spirorbin species, coiling direction was an important taxonomic character, often used to dilineate genera and subgenera (*e.g.*, Caullery & Mesnil 1897). Coiling direction fell out of favour as a diagnostic taxonomic character, as discoveries of dimorphic spirorbins were recorded as incidences of *situs inversus* and therefore not indicative of speciation (*e.g,* Knight-Jones *et al.* 1979). However, my findings suggest reports of *situs inversus* may require further investigation: the reversal of coiling, even at low incidences, may represent important genetic differentiation. Thus spirorbin polychaetes are no exception to the observation that morphologically 'unrecognized' species may be more common among widespread, easily identified 'species' than is generally accepted (Packard & Taylor 1997). Reports of *situs inversus* have surfaced in species descriptions from most spirorbin tribes, including the Januini (pers. obs.), which are thought to be exclusively dextral (Knight-Jones, Knight-Jones & Kawahara 1975). Only the Pileolariini appear to have no reports of *situs inversus,* being exclusively sinistral (Knight-Jones *et al.* 1979).

Nonetheless, even if coiling direction remains a useful taxonomic character at the species level, we are still left with little morphological variation to discern species reliably (Knight-Jones & Fordy 1979). In these cases, molecular taxonomy is an important tool. Although morphological characters remain more convenient, we often do not know which ones are reliable. Thus some investigation into evolutionary relationships with molecular data is required. We are fortunate here that coiling direction, one of the few characteristics of *P. vitrea* that can be seen with the naked eye, infers the same relationship as does the cytochrome B sequences.

An integrative approach to taxonomy, using both morphological and molecular data, provides opportunities not only to discern phylogenetic relationships among species not apparent from morphology alone, but also allows tests of phylogenetic significance of individual characters – in this case, one that was once thought to be highly labile. The process of systematics is iterative, and we must not rely on a single-character-approach to taxonomy (Will *et al.* 2005), whether the characters are morphological or molecular.

Molecular data are not always diagnostic. In fact, sometimes they are unavailable: Numerous attempts to amplify the 5' region of cytochrome *c* oxidase 1 (CO1), which has been touted as the "universal barcode" (Powers 2004), have failed. It appears that the 'universal' CO1 primers need correction in these polychaetes, among others (K. Halanych, pers. comm.), a task that was not successful with the spirorbin species used. Nevertheless, no one marker is going to provide all levels of resolution we need. Cytochrome B, despite nearing saturation, provides enough phylogenetic signal to discern that sinistral *P. vitrea* are likely a genetically independent lineage among dextral forms, a finding strongly supported by other data. Meanwhile, we continue to pursue more reliable amplifications for commonly used genes in phylogenetic inference.

5.5 ACKNOWLEDGEMENTS

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CHAPTER 6.

CONCLUSIONS

6.1 EVOLUTION OF MINIATURIZATION

Miniaturization, or the evolution of extremely small body size, is a widespread phenomenon, and examples occur in diverse taxa (Rensch 1948). Even within the phylum Annelida, there are numerous examples, which are usually associated with interstitial environments (Westheide 1984, 1987). The Spirorbinae are a more conspicuous example of miniaturization, due to their ubiquity and distinctive form (Pillai 1970), and they are a frequently noted example of the relation between brooding and small body size (Strathmann & Strathmann 1982, Strathmann *et al.* 1984).

The factors promoting this evolutionary decrease in body size are unknown. However, recent investigations into phylogenetic relations among the Serpulidae suggest that this trend may have begun before the origin of the distinctive spirorbin coil, given that the small-bodied Filograninae are sistergoup to the Spirorbinae (Lehrke *et al.* 2006, Kupriyanova *et al.* 2006) and not the larger-bodied Serpulinae (as proposed by Ten Hove 1984). Thus coiling and miniaturization may be independent.

The miniaturization trend in the Spirorbinae is often thought to be unidirectional; due in part to the assumption that the large-bodied Spirorbinae (with a large number of thoracic chaetigers) are ancestral (members of the Paralaeospirini and Romanchellini;

e.g., Rhzavsky 1997, Knight-Jones & Fordy 1979, Pillai 1970). However, phylogenetic reconstruction with both morphological and molecular data indicates this group - the two tribes are always members of the same clade - are derived within the Spirorbinae, and therefore the large-bodied species represent an evolutionary size increase. Such size increases appear commonly in all spirorbin clades, including the Pileolariini, Circeini and to a lesser extent the Januini (Fig. 6-1). Thus spirorbin evolution does not exclusively proceed towards small body sizes, and towards a loss of thoracic segments as asserted by Pillai (1970) and Knight-Jones & Fordy (1979); in fact, trends of size increase may be more common than size decreases in spirorbin evolution (from the ancestral size, approx. 2 mm spiral diameter; Fig. 6-1). The large number of thoracic chaetigers characteristic of large-bodied taxa such as *Amplicaria spiculosa*, *Protolaeospira* spp. and *Paradexiospira* spp. may therefore represent an evolutionary gain.

The consequences of miniaturization are highly variable, and depend on the ontogenetic processes involved (Hanken & Wake 1993). It involves not only decrease in body size, but effects on anatomy, physiology, ecology and life history (Peters 1983). It is often associated with structural simplification, morphological novelty, and increased morphological variability (Hanken & Wake 1993). It is also associated with a reduction in fecundity and an increase in egg size (Schultz *et al.* 1991, Shine & Greer 1991) Thus a reversal towards increasing body size may increase fecundity. Indeed, the body size increase within lineages (Cope's Rule) is more common than size decrease (Newell 1949, LaBarbera 1986).

In spirorbins, the commonness of size increase (occurring in a number of clades) suggests selection for higher fecundity; however, these are still exceptions, as small size is still the norm in most clades (most species never exceed 1-2 mm in spiral diameter). Thus there must be still some advantage to staying small; in the Spirorbinae advantages may include adaptations to flexible substrates and other specialized niches (Daly 1978), size refuge from predators, or the ability to have early sexual maturation in unpredictable or ephemeral substrates.

As with many questions in ecology and evolutionary biology, causality is difficult to ascertain (Peters 1983); and in miniaturized species this is often complicated by a 'streamlined' morphology that is a difficult to imagine as a target of selection (Hanken & Wake 1993). Furthermore, the subsequent evolution within a clade may obscure the evolutionary process originally responsible for size decrease.

Miniaturization is often touted as a progenetic process (early sexual maturation, *e.g.*, Westheide 1987). However, other processes may lead to small adult body size. For instance, the idea proposed by Matsuda (1987) seems wholly appropriate when invoked to explain miniaturization in the direct-devleoping Spirorbinae. He suggests that environmentally-induced increase in egg size leads to the incorporation of juvenile stages into the developmental (in this case, brooding) period; thus hatchlings are in many ways are structurally adult, and provide an easy transition to miniaturization.

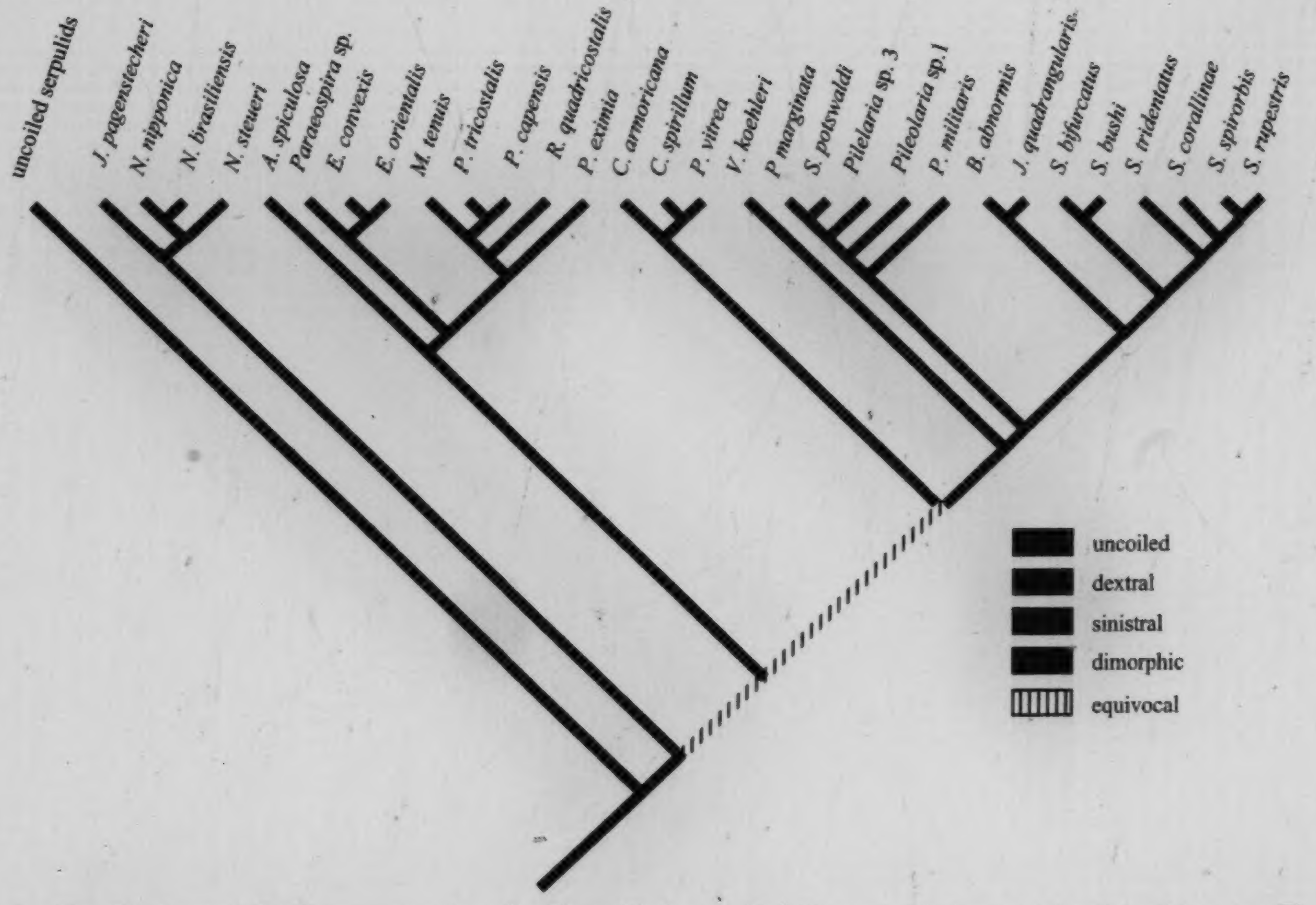
uncoiled serpulids
J. pagenstecheri
N. nipponica
N. brasiliensis
N. steueri
A. spiculosa
Paraeospira sp.
E. convexis
E. orientalis
M. tenuis
P. tricostalis
P. capensis
R. quadricostalis
P. eximia
C. armoricana
C. spirillum
P. vitrea
V. koehleri
P marginata
S. potswaldi
Pilelaria sp. 3
Pileolaria sp.1
P. militaris
B. abnormis
J. quadrangularis.
S. bifurcatus
S. bushi
S. tridentatus
S. corallinae
S. spirorbis
S. rupestris
uncoiled
dextral
sinistral
dimorphic
equivocal

Fig. 6-1 Ancestral state reconstruction of coiling direction on 18 + 28s + morphology (total evidence) tree (see Fig. 3-4).

6.2 EVOLUTIONARY RELATIONS AMONG THE SPIRORBINAE

6.2.1 Evolution of brooding modes

Earlier hypotheses of spirorbin evolution (*e.g*, Pillai 1970, Knight-Jones & Fordy 1979, Knight-Jones 1981) lacked a rigorous phylogenetic basis, so the validity of claims about evolutionary tendencies remained dubious. Phylogenetic analyses of both morphological (Chapter 2) and molecular (Chapter 3) data finally enable the formation of rigorous and novel hypotheses regarding the evolution of spirorbin traits.

Brooding modes are the most well-known of these traits; indeed, evolutionary trends within the Spirorbinae cannot be discussed without reference to brooding modes, as these traits are fundamental to the division of the Spirorbinae into six tribes (Bailey 1969, Knight-Jones 1978). Because the six tribes (Spirorbini, Circeini, Paralaeospirini, Romanchellini, Pileolariini and Januini) form strongly monophyletic groups or paraphyletic grades, these brooding modes have clearly played in important role in spirorbin evolution.

Contrary to a long history of claims that the opercular brood chamber represents an evolutionary novelty (*e.g.*, Elser 1907, Borg 1917, Bergan 1953, Potswald 1968) and a preliminary phylogenetic hypothesis (Macdonald 2003), opercular brooding is ancestral in the Spirorbinae. Analyses of both 18s and 28s rDNA sequence data (Chapter 3) support this hypothesis. Maximum parsimony analyses of morphological data (Chapter 2) indicate that Circeini (brooding inside the tube in a gelatinous matrix) may be the most basal group, yet both equal-weighting and a more 'subjective' weighting scheme place the Januini (opercular brooders with dehiscent operculae) near the base of the tree, and not in a derived clade with the Pileolariini (the other opercular-brooding tribe; their brood chambers are more permanent). Thus perhaps the unique body plan of the Januini (parallel-sided abdominal chaetae, round spermathecae, and two larval attachment glands, among other traits) may not indicate a highly derived phylogenetic position, as suggested by Knight-Jones, Knight-Jones & Kawahara (1975), but rather a more ancient form.

The basal position of *Amplicaria spiculosa* (18s and 28s sequence data; Chapter 3) was surprising; and yielded further support for the plesiomorphic state of opercular brooding within the Spirorbinae. More importantly, single additional taxa can provide crucial phylogenetic information - rare and unique taxa present an opportunity to find 'missing links' in the spirorbin tree, and therefore may lead to a greater understanding of inter-tribal relationships. Perhaps not all spirorbin polychaetes fit neatly into existing tribes.

The results of this thesis (Chapters 2 & 3) challenge the view the Januini and Pileolariini – the two most speciose tribes that include all opercular brooding Spirorbinae - are closely related. Although their brood chamber structure (Knight-Jones & Thorp 1984) and larval characteristics (Höglund 1951) suggested an early divergence, their distinctiveness was not clear until molecular phylogenetic reconstruction revealed the Januini were basal members of Spirorbinae and the Pileolariini derived (Chapter 3; Macdonald & Rouse *In review*). Thus the relative high species diversity of both the Januini and Pileolariini may have separate explanations. Given that the Januini have slow brood turnover, and possibly low lifetime fecundity because they must replace their opercular brood chamber with each brood, their diversity may simply be a result of their greater evolutionary age (Fig. 3-5) and not simply an advantage of opercular brooding. The Pileolariini, on the other hand, may have circumvented this cost to opercular brooding and therefore taken advantage of the potential benefits, such as increased oxygenation of the brood (Thorp 1975, Thorp & Segrove 1975) or avoidance of predation on adult structures (R. Strathmann pers. comm.)

Despite compelling evidence that the Pileolariini type of opercular brooding is advantageous, we must not overlook the possible advantages to tube brooding. The multiple origins of tube brooders (Spirorbini, Romanchellini + Paralaeospirini, and Circeini all likely to have arisen independently from opercular brooding ancestors (Chapter 3, Fig. 3-5) suggest there must be a selective advantage to brooding inside the tube. Thus we have direction for future comparative studies of spirorbin reproduction: tube brooding may reduce susceptibility to predation on larvae or adult structures, or, more intriguingly, may permit brooding to begin at a smaller body sizes than opercular brooders (Chapter 4).

The results in this thesis have made much headway into increasing our understanding of spirorbin evolution. However, some roadblocks remain: (1) overall morphological homogeneity, combined with important taxonomic characters unique to each tribe, and, (2) the lack of shared genetic characters among tribes, which both decrease the ability to discern basal relationships among spirorbin tribes. Additional morphological and molecular would be helpful. Ontogenetic and larval characters may provide valuable clues (given these traits can be more canalized and conserved. *e.g.,* West Eberhardt 2003, 2005), as would more genes for molecular analyses. One thing is certain: the need for integrative taxonomy is paramount, given the possible occurrence of cryptic species (Chapter 5) and the potential for incongruent hypotheses stemming from different genes (Chapter 3). The construction of phylogenies is an iterative process; and further investigation is always warranted.

Although early hypotheses of spirorbin evolution can be traced through logical and informed thought processes (*e.g.*, Pillai 1970), we cannot escape the fact that these were weak due to their lack of phylogenetic framework. Yet these hypotheses become accepted with time. The examination of such evolutionary hypothesis in a phylogenetic framework is of fundamental importance in evolutionary biology. Increasingly, we are finding how to make these phylogenies more robust, and therefore our hypotheses about novel forms.

6.2.2 Phylogenetic reconstruction

The tools we use to construct phylogenies are constantly evolving. Associated with this rapidly-evolving field are vigorous debates about certain procedures such as the combination of data (*e.g.*, Kluge 2004, Naylor & Adams 2003, O'Leary *et al.* 2003), the relative value of morphological and molecular data (*e.g.*, Hebert *et al.* 2003, Miller *et al.* 1997, Lindgren *et al.* 2003) and appropriate character weighting schemes in parsimony methods (*e.g.*, Barker & Lanyon 2000, Cunningham 1997).

Data combination is an important topic of discussion in recent years as molecular techniques advance and data from multiple sources (*e.g.*, muliple molecular markers) become more readily available. The term 'total evidence' has come to mean 'simultaneous analysis' in some cases (perhaps not in the spirit intended by Kluge (1989); Kluge 2004), and may lead to the inclusion of incongruent and misleading data

(Lecointre & Delaporte 2005). However, if we take the meaning of the term in spirit, and assess separate analyses on their own merit (as in Chapters 2 & 3) we can begin to understand what data are misleading, and assess patterns among various data sets. Corroboration, although not 'support' in the statistical sense, is more direct evidence of support for a given hypothesis (Egan 2006).

In the spirorbin phylogeny, certain branching events seen in numerous analyses are intuitively better 'supported' – namely the basal position of the Januini (opercular brooders with a disposable chamber), the sister relationship of the Paralaeospirini + Romanchellini (brood loose in tube and on a thoracic attachment stalk respectively), and the multiple origins of tube brooding (Chapter 3), which are common among analyses of morphology and molecular (18s and 28s) data. However, some branching events, such as the sister group relation between the Pileolariini (reusable opercular brood chamber) + the Spirorbini (embryos brooded in a string inside the tube), the position of the Circeini (brood in a gelatinous matrix), and the position of the enigmatic *Amplicaria spiculosa* (unique opercular brooder) are not the same in all analyses, and therefore need further corroboration.

Assessing the relative importance of molecular and morphological data when looking for corroboration among data sets is not an easy task. Most authors support the use of both data types in forming evolutionary hypothesis, and recognize each has utility (*e.g.*, Miller *et al.* 1997, Baker *et al.* 1998, Baker & Gatesy 2002). A purely molecular approach (*e.g.*, Hebert *et al.* 2003) does not find support in this study, as morphology plays an important role in our understanding of spirorbin relationships: the use of morphological data allows for greater taxon sampling (and use of a large collection of spirorbin polychaetes preserved in formalin). It also allows the inclusion in the analysis of rare and crucial taxa (*e.g.*, the antisymmetric *Neomicrorbis azoricus*).

Nevertheless, molecular data can provide information that morphological data cannot. For instance, they provide a large number of characters (nucleotide base pairs), potential for many 'independent' data sets for corroboration (genes) and can detect hidden variation. In Chapter 5, molecular data revealed the sinistral *Paradexiospira vitrea*, although virtually identical morphologically to the dextral form (except, of course, for coiling direction) formed a distinct clade among dextral members of the species. This result was surprising, and made further investigations into ecological isolation possible.

Character weighting schemes have also been much discussed in the literature (*e.g.*, Farris 1969, Wheeler 1986, Williams & Fitch 1989, Cunningham 1997 and references therein). In the construction of the morphological phylogeny (Chapter 2), it was helpful to weight characters based on a 'subjective' weighting scheme (weighting those characters used by taxonomists more heavily), as this topology turned out to be the most congruent with the molecular hypotheses. Indeed, weighting increases phylogenetic accuracy in simulations and 'known' phylogenies (*e.g.*, Bull *et al.* 1993, Cunningham 1997). When assessing the relative impact of different weighting schemes (a sensitivity analysis is essential) to determine if the data are weakly structured (Barker & Lanyon 2000), and therefore subject to topological lability. This apparently was the case in Chapter 2 (morphological data), as the weighting scheme greatly affected the inferred topology, but none of these trees were strongly supported. Molecular data proved useful in improving this resolution (Chapter 3) and provided independent tests of the hypotheses generated in Chapter 2 and Macdonald (2003).

6.3 EVOLUTION OF ASYMMETRY

Genetic assimilation (phenotype-precedes-genotype; Waddington 1953) is a controversial and poorly understood evolutionary process. Its existence is debated (*e.g.*, West Eberhardt 2005), but is supported by ancestral state reconstruction of conspicuous bilateral asymmetries in many taxa (*e.g.*, the priapium of phallosththid fish, Parenti 1996; crusher claws in some brachyuran crabs, Spears *et al.* 1992; see Palmer 1996 for an exhaustive list) as their directional bias appears to have arisen by way of an antisymmetric precursor where direction of asymmetry is not inherited (Palmer 2004). Spirorbin tubeworms support this pattern as well; the basal position of the antisymmetric *Neomicrorbis azoricus* Zibrowius 1972 (with random direction of coiling) confirms that this may be a possible intermediate on the route to the directional asymmetry (consistent bias towards a particular side) seen in most extant spirorbin polychaetes.

Unfortunately, the inference of ancestral states in spirorbin tubeworms depends heavily on the inclusion of rare, monotypic taxa such as *Neomicrorbis azoricus*. For instance, the hypothesis that directional coiling arose through genetic assimilation of an antisymmetric (random-coiled) precursor depends entirely on the basal phylogenetic placement of *N. azoricus* (the only known spirorbin polychaete that apparently exhibits true antisymmetry). This placement, inferred from morphological data (Chapter 2; Macdonald 2003) should be corroborated with molecular data. Unfortunately, this species is difficult to acquire due to its abyssal distribution (~2000 m deep in the North Atlantic) and obtaining appropriately preserved tissue for molecular work has proven difficult. This is indeed unfortunate, as ideally we would like to test whether the coiling morphs are distinct genetic lineages as in *Paradexiospira vitrea* (Section 5.4.1).

Studies of genetics and ontogeny offer additional insights into the evolution of directional asymmetry in spirorbin polychaetes. An understanding of the relative impact of genetic and environmental factors in the evolution of asymmetry requires consideration of these ontogenetic patterns as well as phylogenetic ones (Palmer 2004). Comparative developmental studies of dextral, sinistral and potentially dimorphic species may give us clues about the interplay of cytogenetic and epigenetic effects in the determination of coiling direction, from the direction of early cleavage to the development of larval musculature. Indeed, the pathways to dextral and sinistral forms may not be mirror images of each other, as in the snail species *Lymnaea stagnalis* (Shibazaki *et al.* 2004). In either case, an understanding of the embryonic and larval development of spirorbin polychaetes may help us better understand how developmental processes effect evolutionary change.

6.4 FUTURE RESEARCH

Spirorbin tubeworms are a unique group of organisms, being a well-defined monophyletic group with intriguing life history and developmental traits. Exciting avenues for research remain, and these bear mentioning, given these diminutive tubeworms have historically been overlooked in studies of ecology, evolution and development but have great potential as model systems in may fields of biology.

6.4.1 Ecological interactions

To understand the origin of the unique body plan of the Spirorbinae, they must be placed in an ecological context. However, their ecological role(s) and interactions remain enigmatic (Chapter 1). Adaptive explanations for their coiled tubes, asymmetric bodies and miniaturized body plan are not obvious, although it seems these must exist given their diversity and wide distributions.

The selective pressures acting on spirorbin polychaetes remain conjecture. Most studies of the Spirorbinae have focused on taxonomy (the majority; see work by P. & E.W. Knight-Jones, P.J. Vine, A.R. Rhzavsky), morphology (*e.g.,* Rhzavsky 1994, Knight Jones & Fordy 1979, Beckwitt 1981, Knight-Jones & Thorp 1984), larval behaviour (Höglund 1951, Knight-Jones 1951, Gee & Williams 1962), and reproduction (Bailey 1969, Hess 1993, Potswald 1967, 1968, Kupriyanova *et al.* 2001, Macdonald 2003). Little is known about their interactions with other organisms, be they predators, plant or animal hosts (on which spirorbins settle), or competitors.

An understanding of their interactions with other organisms, or lack thereof, may enable us to better understand not only their diversity, but also the origin and maintenance of their unique body plan. For instance, investigations into the identity and behaviour of their predators may reveal a reason for the maintenance of their asymmetric body plan. These may also shed light on predation pressure experienced by the various brooding modes and perhaps provide an explanation for phylogenetic patterns observed in Chapters 2 and 3.

6.4.2 Biogeography and speciation

The Spirorbinae seem to lend themselves especially well to biogeographic studies. Their short-lived larval stages and associated limited dispersal (*e.g.*, Knight-Jones 1951, 1953) present an opportunity to test assumptions about symapatric speciation. For instance, restricted dispersal may promote the effects of local selection, inbreeding and drift (Slatkin 1985; Lambert *et al.* 2003). It may also promote the persistence of *situs inversus* in the populations of dextral spirorbins where it is so prevalent. The discovery that the sinistral from of *Paradexiospira vitrea* is genetically distinct from the dextral ones (Section 5.4.1) may only be the beginning of understanding the substructuring of populations of many spirorbin species, whether they are dimorphic or not.

Many spirorbin species are thought to be cosmopolitan (*e.g.*, Knight-Jones & Fordy 1979, Knight-Jones & Knight-Jones 1984, Knight-Jones *et al.* 1991). However, the genetic divergence we see in the morphologically similar dextral and sinistral *P. vitrea* (with the exception of coiling direction) calls into question their status. Whether widespread species are really only one species (*e.g, Janua pagenstecheri* and *Circeis spirillum* are both thought to have a worldwide distribution), or perhaps an assemblage of many cryptic species, remains unknown.

Coiling direction is not the only correlate of genetic divergence of interest in the Spirorbinae. Compelling examples of possible ecological isolation exist not only among sister species of Spirorbinae but also within the same species. My study of *Paradexiospira vitrea* shows how a similar morphological difference reflects a deeper genetic and ecological divergence (Section 5.4.1). There are other examples: *Circeis spirillum paguri* is a form of *C. spirillum* that lives exclusively on hermit crab shells

(Knight-Jones & Knight-Jones 1977). *Spirorbis corallinae* is virtually identical to *S. tridentatus* except that it lives exclusively on coralline algae whereas *S. tridentatus* is more cosmopolitan (Knight-Jones & Knight-Jones 1977). The Pileolariini contain a number of species "complexes" that contain numerous very similar species that differ in their substrate choice and operculum morphology, and that may or may not be related (Rhzavsky & Knight-Jones, in prep.). In Barkley Sound, *Circeis armoricana* is common on many macroalgae, from small, subtidal and filamentous red algae, to large kelp blades floating on the surface; if larvae settle preferentially on certain species and not on others this would suggest additional cryptic species. The list goes on; much raw material exists here for understanding how sympatric speciation has occurred, and is occurring.

6.4.3 Evolution of tube coiling

A coiled calcareous tube is the most obvious characteristic of the Spirorbinae, and the one for which they are named. Thus it seems appropriate to end with a discussion about its origin. A recent ultrastructural study (Taylor & Vinn 2006) suggests that fossils resembling spirorbin polychaetes that date back to the Ordovician (many classifed as *Spirorbis* species) may actually be an order of lophophorates, the Microconchida. This group is now extinct, and the authors suggest spirorbins now occupy this niche. Because this body plan evolved convergently twice, the following question arises: why is this body plan successful?

Daly (1978) proposed several attractive advantages to tube coiling: the ability to limit flexion stress, and therefore to invade algae substrates, the continuous renewal of adhesive area, and the protection of inner thin-walled whorls. Other possibilities include closer packing in high-density situations, decreased encounters with predators, or perhaps a combination of any of these.

No examples of uncoiled spirorbin polychaetes are known (except perhaps the genus *Helicosiphon*, which is elongated but retains a corkscrew shape). This may be the result of phylogenetic and developmental constraints. Some serpulids coil occasionally (*e.g.*, *Pomatoceros triqueter* and *Pseudochitinopoma occidentalis*, among others; Ten Hove 1984, pers. obs.); perhaps here lies a clue to the benefits of a coiled tube. Ecological correlates of the incidence of coiling in these species may yield clues as to why it persisted in the Spirorbinae but not in other serpuliform polychaetes.

Tube coiling in general may be yet another example of how phenotypic plasticity can give rise to novel forms (West-Eberhardt 2005, Palmer 2004): perhaps coiling itself was a response to an environmental variable that became genetically fixed over time. For instance, if the proto-Spirorbinae was an algae dweller (unclear from these phylogenetic studies), then perhaps those individuals with coiled tubes had better adhesion due to flexion stress of renewal of adhesion area. Perhaps the coiling phenotype, and not just the direction of coiling, underwent genetic assimilation (or another evolutionary process) to give rise to the modern Spirorbinae.

Although coiling itself is mysterious; the advantage of one direction over another is even more baffling. Sinistrality seems to have benefits over dextrality: Not only are most of the Spirorbinae sinistral (Knight-Jones & Fordy 1979), but reversals are rare, if nonexistent, within sinistral species and clades (*e.g.*, Romanchellini + Paralaeospirini, Pileolariini) (Fig. 6-2). Dextral Spirorbinae, on the other hand, commonly exhibit *situs* *inversus*, and are members of mixed clades (dextral and sinistral species; Spirorbini, Circeini, Januini; Chapter 3). For some reason sinistrality appears to be evolutionarily more stable. An explanation for this phenomenon may lie in their development.

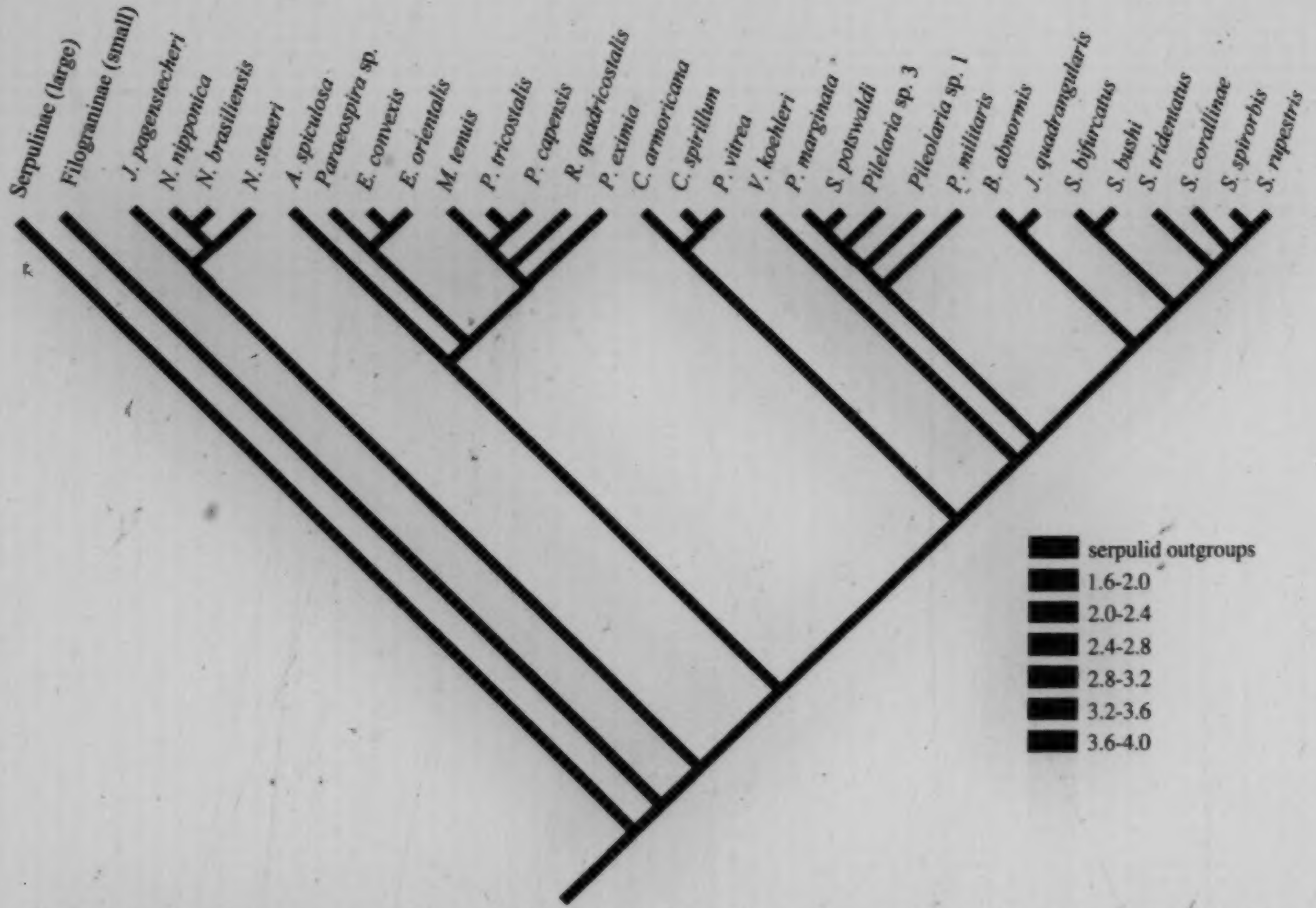
Serpulinae (large)
Filograninae (small)
J. pagenstecheri
N. nipponica
N. brasiliensis
N. steueri
A. spiculosa
Paraeospira sp.
E. convexis
E. orientalis
M. tenuis
P. tricostalis
P. capensis
R. quadricostalis
P. eximia
C. armoricana
C. spirillum
P. vitrea
V. koehleri
P. marginata
S. potswaldi
Pilelaria sp. 3
Pileolaria sp. 1
P. militaris
B. abnormis
J. quadrangularis
S. bifurcatus
S. bushi
S. tridentatus
S. corallinae
S. spirorbis
S. rupestris
serpulid outgroups
1.6-2.0
2.0-2.4
2.4-2.8
2.8-3.2
3.2-3.6
3.6-4.0

Fig. 6-2 Ancestral state reconstruction of body size (spiral diameter, mm; a continuous character) on the 18s + 28s + morphology (total evidence) tree (see Fig. 3-4).

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